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It is with great appreciation that the officers and members of the Society for the Study of Development and Growth acknowledge the aid and co-operation of all who contributed so generously to the success of the 1948 Symposium. Particular thanks is due the University of Vermont for its cordial hospitality.

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THE STUDY OF GROWTH AND METABOLISM BY MEANS OF SPECIFIC ANTAGONISTS

D. W. WOOLLEY

Rockefeller Institute for Medical Research

The fact that growth and other aspects of development are directly under the control of a series of specific chemical substances, or metabolites, will come as no surprise to members of The Society for Development and Growth, for you have been cognizant of it for a long time. When an organism is deprived of one of these essential substances, a more or less specific interference with some phase of metabolism results, and this is reflected in abnormalities of growth or development in immature individuals, or in other functions in adult ones. A classical way of doing this has been to remove a gland or organ which is the manufacturing site of a given hormone, and to observe the results of this deprivation. These can usually be shown to arise from the deficiency of one, or at most a few, chemical substances, because the injection of the latter restores normal function.

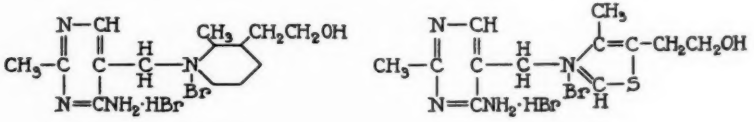
A second way in which specific deficiency of a metabolite may be produced in an organism is to withhold the needed compound from the food, in other words, to create a vitamin deficiency. This second method has greatly increased the range of species which may be studied, because by means of it, minute microorganisms as well as higher animals, are open to investigation.

A third method of causing specific deficiencies of a single metabolite has been used to advantage by Dr. Beadle and his associates, who injure an organism by induction of a mutation with radiant energy. Such mutants lack the ability to form an essential substance, and must now depend on the outside environment for it. One may say that the metabolite which in the parent strain was possibly a hormone, has, by the induced mutation, become a vitamin for the derived strain. Microorganisms are particularly adapted to this type of experimentation, because of their frequency of generation. Aside from the genetic considerations one may say that with Neurospora one achieves by

radiation the essential result which, with a mouse, one gets by surgery.

For the past decade we have been working with yet a fourth method of specifically depriving an organism of an essential metabolite, and thus interfering with some phase of development or function. I would like to tell you something about it. The method consists of administration of a chemical compound related closely in structure to an essential metabolite, but not quite identical with it. By a subtle interference with the action of the metabolite, a specific deficiency of it is produced. The metabolite may be a vitamin or hormone or amino acid or other type of essential substance, and the whole range of living things, from higher animals to bacteria, may be employed. Indeed, as I hope to show presently, even the submicroscopic viruses have been used, and some of their secrets scrutinized.

Let us examine a few examples of this phenomenon of antagonism between metabolites and their inhibitory structural analogs. If one replaces the S atom of the vitamin thiamine with two C atoms, pyrithiamine is produced (Figure 1). If this latter substance is given to



Pyrithiamine Thiamine

FIGURE 1

an animal such as a mouse, the train of signs which is seen in thiamine deficiency is called forth. Growth is retarded, and the convulsions and other manifestations of neurological change as seen in the deficiency disease are produced. All these signs may be erased, or prevented, by feeding more of the vitamin along with the drug. In other words, the toxicity of pyrithiamine does not depend solely on the dose, but rather is directly proportional to the thiamine intake. When the ratio between analog and vitamin exceeds a limit which we may call the inhibition ratio or index, the characteristic signs of thiamine deficiency result. We may say that the two compete with each other in the organism. A few data to illustrate these points are shown in Figure 2.

Pyrithiamine produces retardation of growth in many micro-

Response of Mice to Various Doses of Thiamine and Pyrithiamine

Pyrithiamine	Thiamine	Animals Showing Deficiency Signs	Average Change in Weight
gamma per day	gamma per day	per cent	gm. per wk.
0	1.6	0	+3.0
600	1.6	100	-0.2
300	1.6	100	+1.9
100	1.6	100	+2.5
100	2.0	75	+2.4
50	2.0	0	+3.5
600	61.6	0	+3.1
2000	60.0	0	+3.6

FIGURE 2

organisms as well as in animals. This effect may be ascribed to thiamine deficiency since withholding of that vitamin from the nutrient solution has the same effect. An even stronger reason for saying so is that additional amounts of thiamine completely overcome the toxicity of pyrithiamine. Figure 3 will show the relative effectiveness of pyrithiamine against a variety of species. Here one can readily see that the analog affects only those forms which have a nutritional requirement for the vitamin. If the microorganism can synthesize its own thiamine, pyrithiamine will not prevent it from growing. This dependence of action of the analog on the nutritional needs of the organism is a common phenomenon in the study of structural relatives of vitamins, although it is not a universal finding.

Another well-known example of competition between a vitamin and structural analogs of it is that of p-aminobenzoic acid and the sulfonamide drugs. This was discovered by D. D. Woods in 1940. You are all familiar with the fact that p-aminobenzoic acid is an essential metabolite for bacteria, and that deficiency of it, with consequent in-

Inhibitory Power of Pyrithiamine for Various Microbial Species

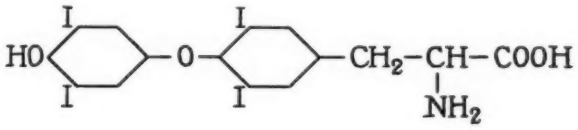
Organism	Inhibition index $\frac{\text{Pyrithiamine}}{\text{Thiamine}}$	Thiamine requirement
Ceratostomella fimbriata	7	Intact thiamine
Ceratostomella from London plane tree	19	" "
Ceratostomella pennicillata	10	" "
Phytophthora cinnamomi	12	" "
Chaloropsis thielavoides	11	" "
Endomyces vernalis	130	Pyrimidine
Mucor ramannianus	800	Thiazole
Saccharomyces cerevisiae	800	Pyrimidine and thiazole
Staphylococcus aureus	2000	" "
Salmonella gallinarum	1000	"
Neurospora crassa	Greater than 400,000	None
Escherichia coli	" " 2,000,000	"
Clostridium butylicum	" " 2,000,000	"
Lactobacillus arabinosus	" " 40,000	"
Lactobacillus casei	" " 5,000,000	"
Lactobacillus delbruckii	" " 5,000,000	"
Lactobacillus mesenteroides	" " 5,000,000	"
Lactobacillus pentoaceticus	" " 5,000,000	"
Streptococcus lactis R	" " 5,000,000	"
Propionibacterium pentosaceum	" " 5,000,000	"
Hemolytic streptococcus H69D	" " 4,000,000	"

FIGURE 3

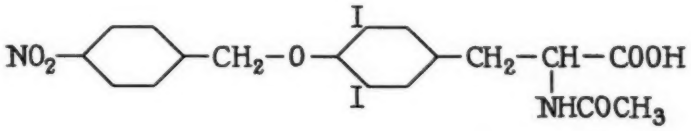
hibition of growth, is produced by the sulfonamides which have a close structural resemblance to it. Thus far, no one has been able to cause deficiency of p-aminobenzoic acid in animals with these analogs, but neither has such a deficiency disease been evoked by any other method in higher animals.

For every one of the water-soluble vitamins, and for many of the fat-soluble ones, structural analogs have been produced which will call forth in living things the signs of deficiency of the metabolite to which they are related. A catalog of these would more than fill the hour. Rather than enumerate this list, let us see how the same phenomenon may be found with metabolites other than vitamins, and more especially, let us turn to a few examples of the usefulness of the knowledge in the study of metabolic functioning.

One of the hormones which has been converted by slight structural alteration into an antagonistic agent is thyroxine. When small amounts of this hormone are administered to tadpoles, a marked acceleration of development takes place, so that an animal which would develop into a frog in about a year, is caused to change into a miniature adult within 2 weeks. The change is so rapid that, unless the dose is very small, the tadpoles die from the strain. This increase in the metabolic rate, with its attendant acceleration of development, should be overcome by an effective structural analog of the hormone. Such analogs have been synthesized, and the structure of one of them is shown in Figure 4. When this drug is given to tadpoles along with a lethal dose



Thyroxine



p-Nitro benzyl ether of N-acetyl diiodotyrosine

FIGURE 4

of thyroxine, the animals are protected from death. The acceleration of metamorphosis is very greatly reduced, but it is not entirely eliminated. The specimens shown in Figure 5 will give some indication of this.

The reason why complete nullification of the action of thyroxine could not be achieved probably resides in the finding that large doses

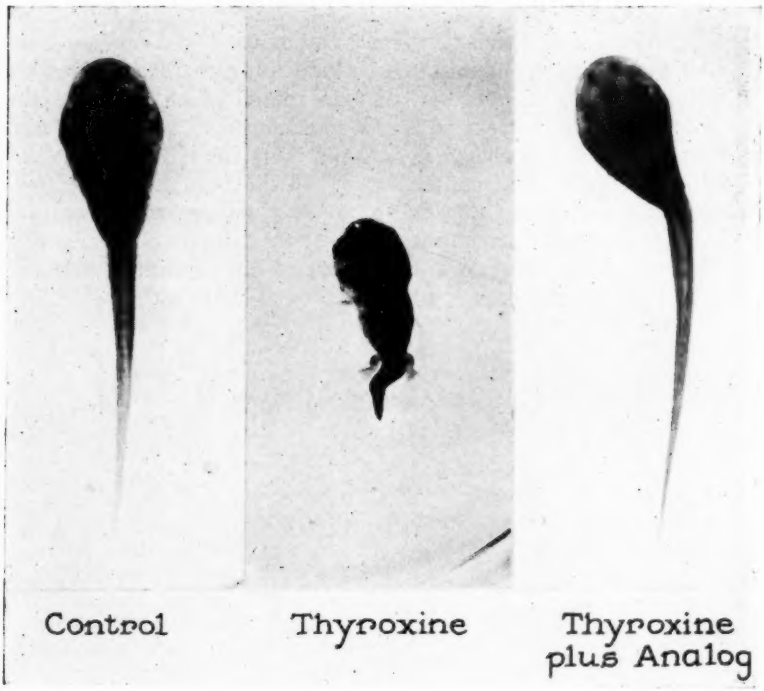


FIGURE 5

of the analog by themselves had some slight thyroxine activity. As the dose is raised in an effort to overcome completely the effects of the hormone, the range of thyroxine activity of the analog is reached. Such antagonistic effects of low concentrations of the analog, coupled with pro-metabolite activity of larger amounts, have been found with compounds related in structure to other metabolites as well as to thyroxine. It recalls the fact familiar to all pharmacologists, that changing the dose of certain drugs may almost reverse the character of their manifestations.

Structural analogs antagonistic to various metabolites are not just artificial products of the organic chemist. Nature apparently uses the phenomenon, possibly to control specific processes. Let us consider

the case of the estrogenic and androgenic sex hormones. Here are two compounds, viz. estradiol and testosterone, which are rather close stuctural analogs, and yet with apparently antagonistic actions in an organism. In several functions, such as the deposition of bone minerals, as well as the feminizing and masculinizing properties, this pair of hormones has been shown to be antagonistic to each other. Dr. Hertz has recently examined such relationships in considerable detail. On the other hand, I believe there are situations in which these hormones act synergistically, or do not interact at all. It has long been a puzzle as to why the female sex hormone is found so abundantly in males of certain species, and why the male hormone is present in females. If the expressions of sex are not due to the actions of single compounds, but rather are the resultant of the antagonistic properties of a pair of structural analogs, the explanation of this anomaly might be indicated. In the lower forms of life such as certain algae, Kuhn and his collaborators ten years ago brought forth much evidence that one aspect of sex was controlled not by a single compound, but rather by the interplay of two closely allied substances, cis and trans dimethyl crocetin. Either male or female behavior could be elicited by varying the ratio of these two structural analogs. A detailed consideration of the phenomenon of antagonism between structurally similar compounds would reveal some nice advantages that the organism might possibly enjoy by using such means for control of individual functions. Thus, one or more specific activities of a metabolite might be held in check by a structurally similar metabolite, while other properties of the first one could act unchecked. In this way by varying the individual concentrations of the two substances, an organism would have a wide control over a family of functions. From the work with artificial antagonists produced in the laboratory, it is clearly evident that analogs can be made which will nullify only a portion of the effects of a vitamin or hormone.

Several investigators have found evidence that other pairs of similar metabolites may behave antagonistically in an organism. For example, the porphyrins, and pairs of closely related amino acids and purines have been studied. Dr. Shive and his associates, as well as many others, have examined in this light the relationships between amino acids. There is little difficulty in demonstrating the antagonism. The metabolic interpretation, however, which one should place on all such demonstrations is open to question. In the case of the pairs of

amino acids, for example, detailed investigation has revealed exceedingly complex interrelations which may arise from the fact that certain amino acids are apparently interconvertible metabolically. To the present author it would seem likely that much will be learned by further study of this phase of the phenomenon, but that there is considerable danger of pushing the explanations too far. The use of isolated enzyme systems in place of whole organisms might be of great value in such work. In this way complicated concomitant reactions which proceed in the cell might be circumvented.

Having introduced enzymes into the discussion, let us now consider the biochemical mechanism by which antagonism between structurally similar compounds is believed to operate. This will involve enzymes, or, at least, specific proteins. Currently, two stages are recognized in an enzyme reaction. The first is the combination of the enzyme with the substrate. After this has occurred, a reaction takes place in which the substrate is changed, and the original enzyme is liberated. It may then repeat the process with new substrate molecules. Now, a compound closely related in structure to the substrate can, by virtue of this similarity, combine with the enzyme. Nevertheless, the fit may not be good enough to allow the second stage of the process to occur. In this situation, the enzyme is tied up with a compound it cannot alter, and is unable to combine with normal substrate. Since this substrate or metabolite enters into living processes usually by enzymic reactions, the organism is thus denied its use, and a specific deficiency is produced. The combination of enzyme and substrate is a reversible one, and so, usually, is that of the enzyme and the analog of the substrate. In a mixture of analog and substrate, the one which gets the attention of the enzyme will depend on the relative affinities of the two for it, and on the relative concentrations of the two. If the substrate is in combination, and the concentration of analog is increased, the latter will displace the former from its union. The phenomenon of competition is viewed in this light. The specific protein need not be an enzyme; for example, it may be merely a body which combines more or less specifically with a metabolite. Hemoglobin and its combination with oxygen is a case in point, and here one can see readily the displacement of the oxygen by the structurally similar carbon monoxide.

The mechanism of action of antagonistic analogs which has just been briefly outlined is not an entirely satisfactory one, but is the best

we have. The successes which have attended its use in viewing the phenomenon have been sufficient to justify its continuance until a better one is found. It may even be possible that it is the true explanation.

By taking advantage of the phenomenon of antagonism between structurally related compounds, one may study certain aspects of life not readily accessible by other means. I would like to illustrate this by sketching for you some of the experiments we have done with the influenza virus. I have selected this example with some misgivings because the exact chemical structures of some of the compounds involved are unknown, and therefore the relevance of the whole case may be called in question. However, regardless of the explanation of the facts (i.e., whether they represent another case of competition between structurally similar compounds or not), I hope they are of sufficient interest to those studying growth and development to warrant their discussion.

When the influenza virus attacks a red blood cell, or a susceptible lung cell, it destroys something by what is believed to be an enzymic process. From the work of Hirst we know that the virus causes a suspension of chicken erythrocytes to agglutinate, and that in this process the virus is adsorbed to the cells. As the mixture is incubated, the virus slowly reappears in solution, and the cells may again be suspended. The virus is recovered largely unchanged, but the cells are altered, because they will no longer agglutinate with fresh virus. The result of much study of this phenomenon has been to conclude that the virus contains an enzyme which combines with a specific substrate in the cell and then destroys it. The nature of this substrate is unknown, but Hirst has found some evidence to suggest that it might be carbohydrate. If this be so, then by supplying a suitable structural analog of the substrate, the action of the enzyme in the virus on the cell material might be inhibited. In this system, the substrate in the susceptible cell would be the metabolite, and the added inhibitor would be the analog. Several polysaccharides of plant origin, especially those made up largely of galacturonic acid, were found to inhibit the agglutination of susceptible erythrocytes by influenza virus. Apple pectin was one of the most active of these. Figure 6 will illustrate its effect on hemagglutination. The comparative potency of various polysaccharides is shown by the data in figure 7.

Effect of Apple Pectin on Agglutination of Chicken RBC

in Presence and Absence of Influenza A Virus

Apple Pectin	Hemagglutination	
gamma per cc.	with virus	without virus
6666	partial	partial
3333	partial	none
1666	partial	none
833	trace	none
208	trace	none
104	partial	none
26	partial	none
13	complete	none

FIGURE 6

Effect of Various Carbohydrate-containing Materials on

Hemagglutination by Influenza A Virus

Substance	Inhibitory Activity	Substance	Inhibitory Activity
Polysaccharides		Simple carbohydrates	
Apple pectin	+	Galacturonic acid	0
Citrus pectin	+	Cellobiuronic acid	0
Flaxseed mucilage	+	Inositol galactoside tartrate	0
Gum acacia	+	Galactose	0
Specific polysaccharide of acacia	0	Aldobionic acid of flax	0
Gum myrrh	+	Glucose	0
Alginic acid	trace	Mannose	0
Soluble starch	0	Ribose	0
"Starch polyaldehyde"	0	Complex concentrates	
"Starch polyacid"	0	Blood group A substance	+
Agar	0	Chicken RBC extract	+

FIGURE 7

Effect of Apple Pectin and of Alginic Acid on Multiplication of Influenza A Virus in Embryonated Eggs

Substance	Amount	When Given	Eggs	Eggs Showing Virus Multiplication
None			24	24
Apple Pectin	50	before virus	61	5
		after virus	56	17
	25	before virus	4	1
Alginic Acid	50	before virus	16	14
		after virus	28	24

FIGURE 8

Not only will apple pectin inhibit the agglutination of erythrocytes by influenza virus, but it will also markedly retard the multiplication of the virus in a susceptible host such as embryonated eggs. Data to illustrate this point are shown in Figure 8. You will note that alginic acid, a polysaccharide physically similar to apple pectin, but which was rather inactive as an inhibitor of hemagglutination, likewise did not prevent the multiplication of the virus. The pectin was quite harmless to the growth and development of the embryos in the eggs.

The importance of this selectivity of action cannot be overemphasized when one thinks about chemotherapy. If an analog is active in causing deficiency in the host as well as in the parasite, its ultimate practical value is questionable. What is needed is a selective agent which does not harm the host. Apple pectin seems to be one of these. Another, which may be cited, is phenyl pantothenone. This analog of the vitamin pantothenic acid is highly selective in that it affects microorganisms but not higher animals. Consequently its development and use as an antimalarial drug hold promise. More must be learned about the reasons for selective action of these analogs before a fundamental advance in practical use of them can be made.

These results with pectin have been extended to another virus which reacts with a polysaccharide-like substrate in the susceptible cells, namely bacteriophage. In this case too the cells could be protected

against the virus by an inhibitory polysaccharide of plant origin. Citrus pectin was quite effective in this virus-cell relationship, whereas apple pectin was the best for the influenza system.

Thus, by simple application of the ideas we have been discussing, agents have been discovered which will protect living cells against attack by certain viruses. In the case of the influenza virus, multiplication of the submicroscopic organism was inhibited. Prior to these investigations, no agents which would do these things for any virus were known. In this statement, antibodies must be excluded.

If the way of looking at the attack of the red cell by influenza virus be correct, and if the postulated mode of action of the pectin is right, then the system which has been inhibited by apple pectin should provide a good assay with which to attempt the isolation of the virus substrate in the cell. By increasing the concentration of this substrate in the erythrocyte-virus-pectin system, the effects of the pectin should be overcome, just as the increase of thiamine in the case of a cell inhibited in growth by pyrithiamine restored normal function. This has been done, and it has been found that from susceptible cells, but not from others, a material may be extracted which competes with pectin in the erythrocyte-virus system. Because it seemed probable that this material was the one with which the virus first reacted in the cell, attempts were made to purify it, and it was obtained as a non-dialyzable, nitrogen- and polysaccharide-containing concentrate. It had high activity in reversing the action of pectin. The preparation was undoubtedly not a pure compound. However, its activity was destroyed by reaction with highly purified influenza virus, and during this reaction, a decrease in viscosity occurred. It is hoped that studies of this sort will allow the investigation of metabolic reactions of viruses in the complete absence of cells, and thus may lead to an understanding of some of their vital processes.

In view of the usefulness of antagonistic compounds, a knowledge is desirable of how the structure of a metabolite should be changed in order to convert it into an inhibitory analog. From the information now at hand a few generalizations can be made about this matter. These are illustrated by the examples given in Figures 9 and 10. Here it can be seen that if the metabolite is an acid, the exchange of the carboxyl for sulfonic acid or sulfonamide, or for a sufficiently negative ketone grouping will bring about the desired result. If the metabolite

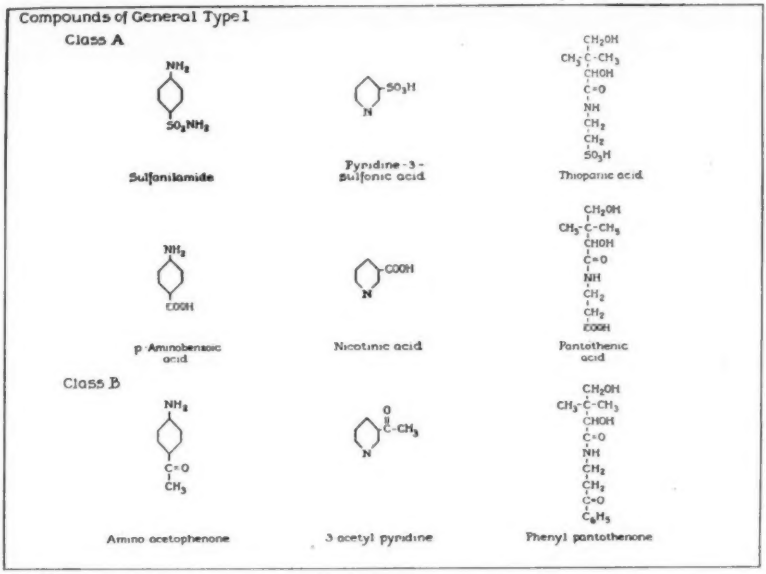


FIGURE 9

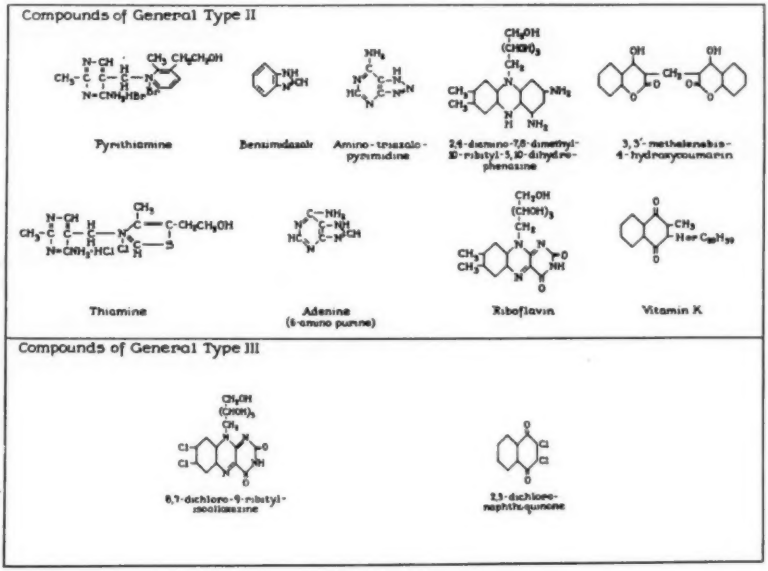


FIGURE 10

is a ring compound, and many important ones are, the trading of one or more atoms in the ring for another atom is frequently a good way to produce inhibitory structural analogs. The atom in the ring may be exchanged for nothing at all, thus yielding open chain analogs and these are sometimes found to be quite antagonistic to the parent metabolite. For example, most of the anti-histamine drugs are related to histamine in essentially this way. Finally the replacement of alkyl side chains with halogen atoms frequently leads to the formation of highly potent inhibitory compounds. There are other ways than these in which the desired result has been achieved, but these seem to be the ones found thus far to be of general applicability. As a rule, one can say that the smaller the alteration in structure has been, the more active the analog will be as an antagonist to the metabolite.

The foregoing summary may give the impression that there is no uncertainty in this matter of predicting inhibitory analogs, and so I hasten to correct this idea. Many of the ramifications of this aspect of the problem cannot be dealt with here, but it must suffice to say that all types of structural change of a given metabolite do not yield agents of identical activity either quantitatively or qualitatively, and that while some analogs cause specific deficiency in a wide range of species, others act against only a limited variety of organisms. Nevertheless, enough empirical information has been gained that an experienced investigator can eliminate much of the chance, and frequently proceed rather directly to a desired end.

METABOLISM IN RELATION TO GROWTH[1]

DAVID R. GODDARD

Department of Botany, University of Pennsylvania

If we consider the inorganic world, we recognize that all processes lead to a random distribution of materials and energy. This finds its expression in the second law of thermodynamics, a law which summarizes the conclusion that every system proceeds in the direction of a decrease in free energy and of maximum entropy. This law is a statistical law, and is valid for large numbers of molecules. Probability considerations make it clear that in a large system observed over a long time, there will be individual isolated fluctuations leading to local temporal decreases in entropy, that is, areas of a higher degree of organization than the average for the system as a whole.

There is no reason to question that the laws of thermodynamics are valid in living systems, though to obtain anything approaching valid proof would be extremely difficult. If there could exist a mind free from a living body, located in interstellar space, such a mind would consider the origin of life as so highly improbable on thermodynamic grounds, as not to be seriously considered. Plants and animals are highly organized systems, containing complex molecules, that of themselves are most improbable structures, to say nothing of the high degree of organization at macromolecular and microscopic levels and at the level of gross morphology. In other words, living systems have a low entropy level. The mere existence of a living cell means that its improbable structure must be maintained through time, that is, it must not proceed toward equilibrium with a decrease in free energy, but the high energy states must be maintained, and it must not proceed to maximum disorganization (maximum entropy) but must continue at a level of entropy below the world level.

If the mere existence of organisms is thermodynamically improbable,

[1]A few of the recent experiments were conducted under the financial support of grants from the following: Sloan-Kettering Institute for Cancer Research and the Cancer Commission of the University of Pennsylvania.

how much more improbable is growth, differentiation, and reproduction? In growth we see the relatively unorganized cell or cells undergo a most orderly process of division and differentiation leading to the more complex organizational level of the adult organisms. As biologists, we recognize that however improbable all of this appears thermodynamically, that it proceeds regularly, and that all fertilized eggs or spores possess the potentiality of ordered growth and development; and that one may chart out in advance the course and time sequences of development of a particular egg with a high probability of success.

We cannot discuss here how the highly improbable event of the first life had its origin, nor how through a period of a billion years the evolution of more highly organized systems evolved from the simplest living forms. But as scientists we must recognize that once life had its origin, the paradox of highly organized systems changing in maturation or in evolution to still more improbable systems of higher levels of organizations, in spite of thermodynamic laws which would appear to lead to death and decay, is more an apparent paradox than a real one. The mere existence of life, growth and evolution, must force us to recognize that growth and evolution are consistent with the laws of thermodynamics, and proceed with a constant increase in the entropy of the whole system. The problem turns on the word "system". If we consider the individual cell or organism as the system, it is true that in its growth and development it proceeds with decreasing entropy. Living organisms are not "closed" thermodynamic systems, and when we recognize the energy system of which the individual cell or organism is but a part, then we can see that the energy system as a whole proceeds in the direction of equilibrium, with decreasing free energy and an increase in entropy. This energy system includes the sun and the nuclear reactions which occur within it, resulting in the constant radiation of energy to the earth. A small part of this energy is trapped by the pigment systems of green plants and stored as the potential energy of the carbohydrates. These carbohydrates serve as the fuel of life, for the green plants themselves and all other living organisms.

In considering then, the energy and organization levels of organisms, in their survival, growth, and reproduction, the organism cannot be considered separately from its food, nor in the last analysis from photosynthetic and respiratory mechanisms and the energy cycles of the sun. The decrease in entropy of growth, then, is more than com-

pensated for by the increase in the entropy of the metabolism of the food, with the result that the entropy of the system as a whole increases. From the point of the energy balance of the universe, life is but a highly improbable fluctuation of microscopic magnitude, but one which possesses the unique property of propagation of this unstable state through time.

Metabolism and Growth

We are lead by these considerations to consider the relation between metabolism and maintenance, and metabolism and growth. If we ignore anaerobic organisms and photosynthesis, all of the energy available to organism must be made available by the oxidation of compounds by molecular oxygen. The individual organism is on an energy slide, a slide which leads to equilibrium with its environment, dispersion of its substance, disorganization of its structure and death. It survives for a limited time on its precarious perch, by counteracting the processes of dispersion and decay by constantly counteracting these processes of breakdown and dispersion by new synthesizes and accumulation, uphill reactions driven by the metabolic engine. There is much discussion of maintenance energy, but little concrete evidence exists as to its magnitude or the reactions involved. However, when we recognize that cells are not in ionic equilibrium with their environments, and that diffusion of substances outward must constantly be overcome by new absorption, that proteins, glycogen, starch, etc. are constantly undergoing hydrolysis, and that the compensating new syntheses require an energy expenditure, maintenance energy has some meaning. Further, it is clear that a cell has an internal organization as well as a specialized surface, the integrity of this structure is not only essential for activity, but separates reactants and resists the movement towards equilibrium. The maintenance of this organization presumably requires some energy expenditure. We do not have sufficient knowledge to calculate the minimum maintenance energy of an organized living cell or organism. The best we can do is to consider the actual metabolic levels which one finds, the "true" maintenance level may be only a fraction of the observed values.

These values seem to vary from a low of about 0.1 μl O_2 per gm. wet wt. per hr. for dormant seeds, to values in the neighborhood of 20-40 for many mature plant tissues and cold blooded animals, to very

considerably higher values for microorganisms and mammals. It is not surprising that dormant structures, which have either a low water content or highly impermeable surfaces may have a very much lower maintenance level than tissues which may readily be shifted into high states of activity. The dormant systems have mechanisms which greatly slow the rate of the slide toward equilibrium.

It is clear that metabolism is an essential condition of growth. There is an abundance of evidence from many sources indicating that in dormant cells and tissues metabolism may proceed at a very low rate, and that marked increases may occur in O_2 consumption associated with the initiation of development. This may be illustrated by some results of Merry and Goddard's (1941) shown in fig. 1. Dormant barley grain of 8.5% moisture has an O_2 consumption of less than 0.1 μl O_2 per hr., this increases some 200 fold in the first hour after the addition of water. Similar changes are associated with dormant spores, as for example the fungus *Neurospora,* where Goddard (1935), Goddard and Smith (1938) have shown that the dormant spore may be stimulated to growth by a heat shock, and following heat treatment, there is a period of high metabolism for 2-3 hours before visible growth may be detected. This is illustrated in fig. 2. As is well known from the results of Warbung (1909), Runnstrom (1933) and others, fertilization of some marine eggs is followed by a considerable increase in metabolic rate. It is true that for some other eggs, no such increase accompanies fertilization. (See the literature summarized by Ballentine, 1940). If the basal metabolic rate is at a sufficiently high level, there may be an adequate supply of energy available for growth, and no over-all increase may be detected when growth is initiated, even though there may be a considerable change in qualitative aspects of that metabolism.

In general, growing and developing tissues show a higher rate of respiration than do adult tissues. This may be illustrated in the results of Machlis (1944) on barley roots, where the majority of the cell divisions occur in the apical few mm. of the root, and the apical portion has a much higher respiratory rate than sections further from the tip. Similar results have been obtained by Berry & Brock (1946) with onion roots, and Ball and Boell (1944) on the apical stem meristem. However, there is one difficulty with this type of result, since the respiratory rate is calculated on a basis of either wet or dry weight,

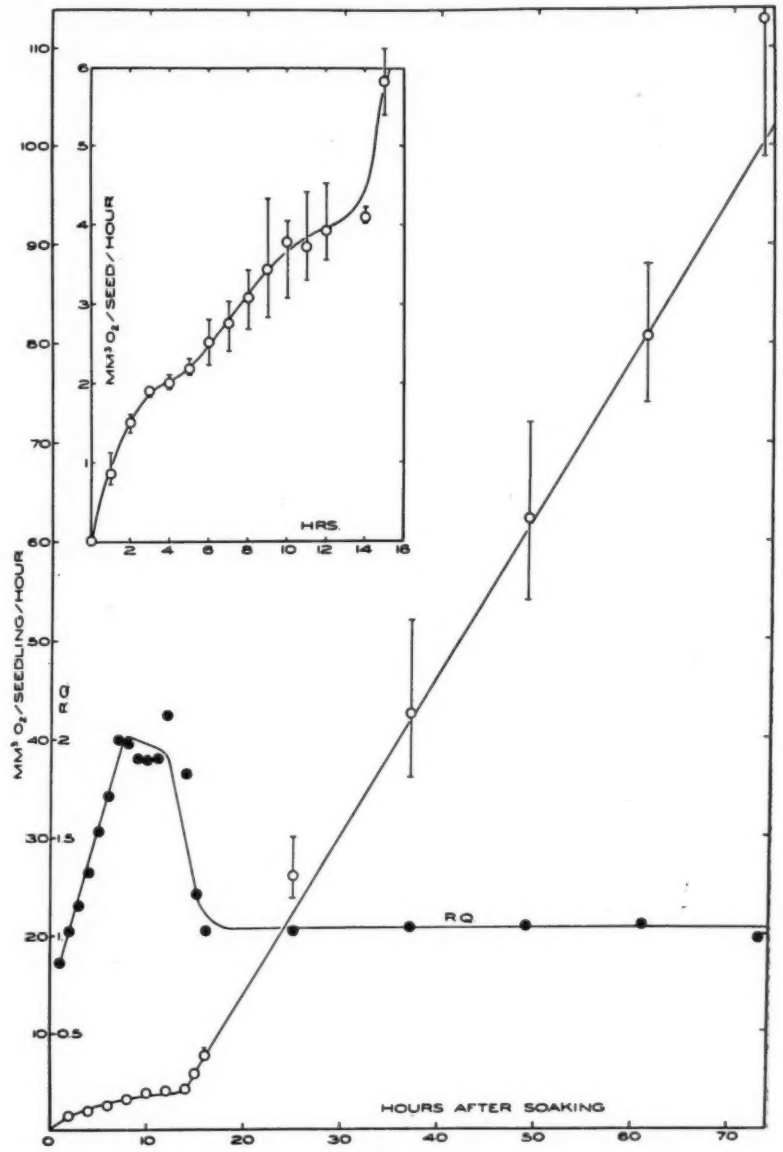


FIG. 1

OXYGEN CONSUMPTION OF BARLEY GRAIN AND SEEDLINGS. SOAKING OF THE DRY GRAIN WAS COMMENCED AT ZERO HOURS, VISIBLE GERMINATION WAS DETECTED AT 14 HOURS. FROM MERRY AND GODDARD (1941).

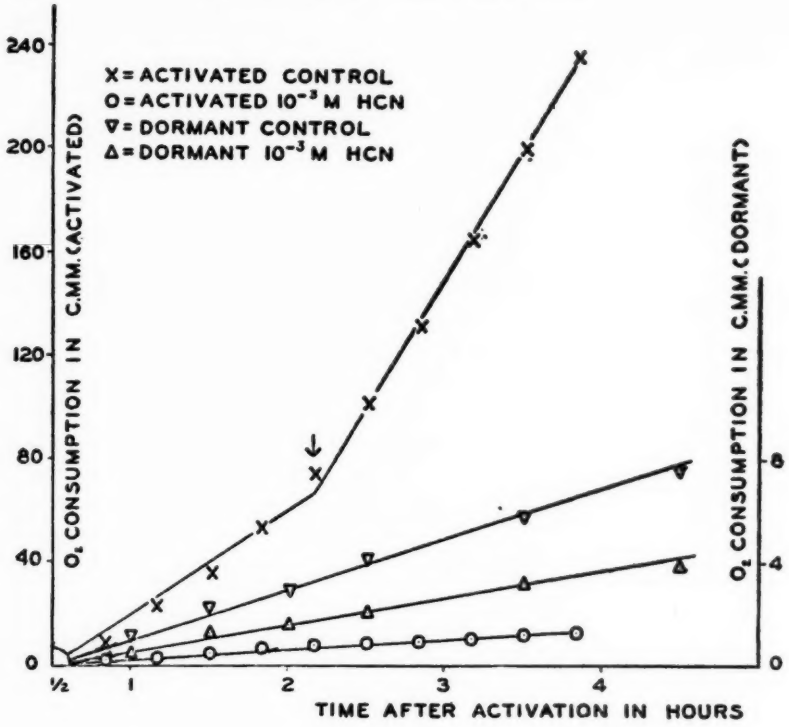


FIG. 2

OXYGEN CONSUMPTION OF DORMANT AND ACTIVATED *Neurospora tetrasperma* ASCOSPORES AT 25° C. ACTIVATION WAS AT 56° C. FOR 20 MINUTES. FROM GODDARD & SMITH (1938).

and the amount of protoplasm may not be at all comparable. Professor Frey-Wyssling in this Symposium reports that if the respiratory rate is referred to the content of extractable nitrogen coagulated by protein precipitants, the results are reversed, and the elongating cells consume more oxygen than do the cells in division. Now that the nucleoprotein contents of tissues may be readily determined, a new representation on that basis may be available shortly. It is interesting to note that Philips (1941) found that embryonic chick tissue had no higher metabolic rate than many adult tissues.

It has long been of interest to know whether respiratory changes accompany cell division. It was early found by Loeb (1895) that under anaerobic conditions cleavage of marine eggs would not be initiated, but Philips (1940) and Brachet (1934) have shown that once cleavage has started, the cell division will go on to completion. Recently Erickson (1947, 1948) has described interesting material in the lily anther. Here, all of the sporagenous cells undergo meiosis and mitosis in a relatively short time period, as is illustrated in fig. 3.

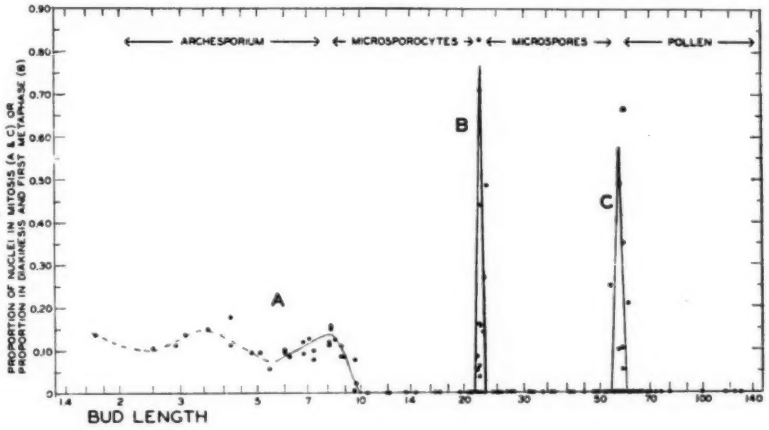


FIG. 3.
PROPORTION OF NUCLEI IN MITOSIS OR MEIOSIS IN LILY ANTHERS. FROM ERICKSON (1947)

Respiratory data, which includes the non-dividing anther wall, as well as the dividing spore mother cells, shows a decrease at division times, as is illustrated in fig. 4. Comparable results have been obtained by Stern & Kirk (1948) and by Zeuthen (1946). It is quite possible that the synthesis of the compounds essential for growth occurs prior to division, and it is even possible that the energy essential for cell division is stored in the pre-division phase, and that division itself is comparable to the relaxing of a stretched spring.

The partial or complete inhibition of respiration by 0_2 lack, or respiratory poisons, is accompanied by cessation of growth. There is no question but that a minimum respiratory rate is essential for

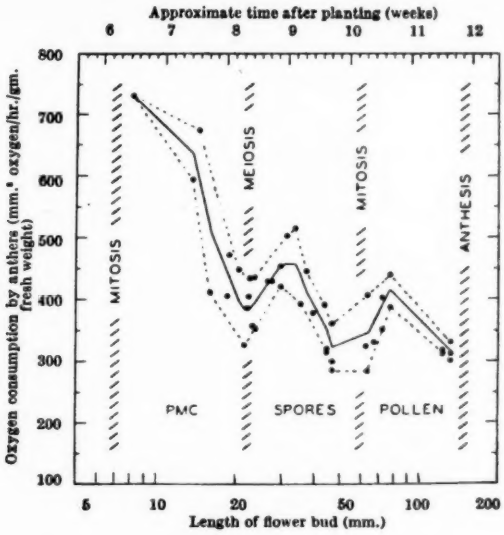


FIG. 4
OXYGEN CONSUMPTION OF LILY ANTHERS. FROM ERICKSON (1947).

growth and development. However, the reciprocal relationship need not be true, that is, growth may be completely inhibited without detectable change in over-all metabolism. Since the work of Clifton (1937) it has been recognized that dinitrophenol and sodium azide at very low concentrations may block oxidative assimilation, that is the conversion of exogenous food into stored reserves, without a decrease in the rate of oxygen consumption. It is clear that these compounds must be effective at some locus in the metabolic scheme, and Loomis and Lipmann (1948) have shown that dinitrophenol prevents the coupling of phosphorylation and oxygen utilization, and this block should be adequate to explain the observed growth results.

We have recently been studying the effect of emetine, berberine, colchicine, nitrogen mustards, and protoanemonin,[1] on cell division and cellular growth. Some results are shown in fig. 5. Of these com-

[1]Dr. Kenneth Savard of the Sloan-Kettering Institute synthesized the protoanemonin and angelica lactones used. We wish to thank him and Dr. Chester Stock for making them available to us.

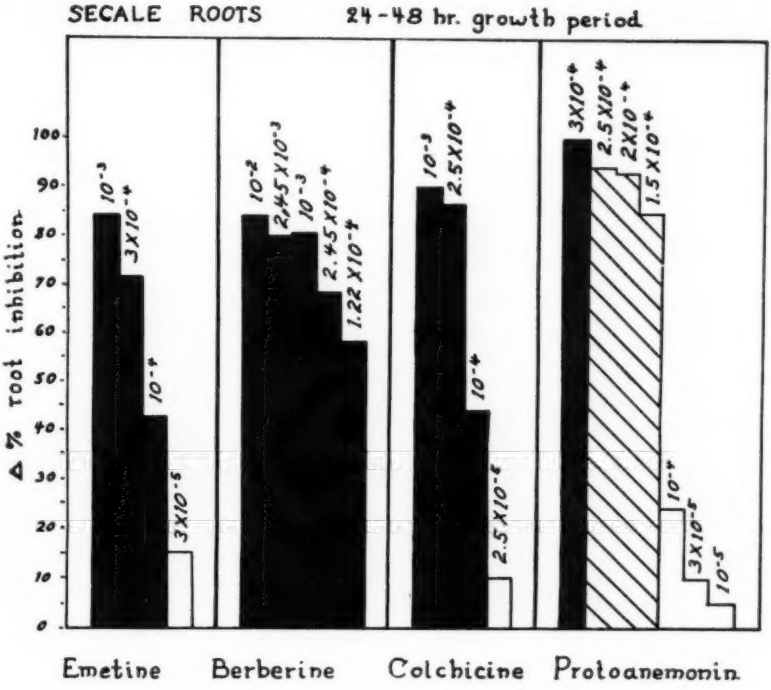
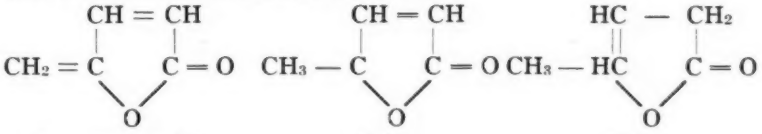


FIG. 5

EFFECT OF CHEMICALS ON GROWTH IN LENGTH OF RYE ROOTS AT 25° C, BLACKED AND CROSS HATCHED AREAS STATISTICALLY SIGNIFICANT. UNPUBLISHED EXPERIMENTS OF GODDARD, C. HOLDEN AND G. ROSEN.

pounds, only protoanemonin blocks O_2 uptake at the same low levels at which it blocks cellular division and growth. This compound is of interest because the closely related compounds, alpha and beta angelica lactone are without effect on growth and oxygen consumption. The structures are illustrated below:



Angelica lactones

Protoanemonin is of particular interest, since Erickson (1948) and Erickson and Rosen (1949) have shown that this compound causes the cytological disappearance of mitochondria and the disruption of the cytoplasm as well as the inhibition of mitosis. Its metabolic effects may be purely secondary to the cytoplasmic disruption. It is known to be an antibiotic against some bacteria and fungi; (Baer, Holden, and Sigal, 1947, and Baer 1948) have reported that it inhibits acetic and pyruvic acid oxidation by intact *Proteus vulgaris,* but only at concentrations which are between 30 to 300 times as high as those which bring about growth inhibition. The mechanism of its action still remains to be explained.

The question of the mechanism of action of antibiotics, such as penicillin and streptomycin is too far afield, but there still seems to be question as to what part of the growth mechanism is blocked by their action.

Qualitative Changes in Metabolism

As embryonic or meristematic areas differentiate in growth, or as growth is initiated in dormant tissues, there may be qualitative changes in the metabolism. We may illustrate this with a few examples from work in my laboratory.

If the dormant spores of *Neurospora* are suspended in 0.005 M iodoacetamide the respiration is inhibited about 45-55%, showing clearly that the poison has penetrated. If these spores are well washed, and heat activated, the results shown in fig. 6 are obtained. It is clear from this graph that the respiration of the activated spores is insensitive to iodoacetamide, but at the time when the control spores begin to germinate the respiration suddenly becomes sensitive to the poison. We have no explanation for these results, but they show quite clearly a qualitative shift in the metabolism associated with the initiation of growth.

There is considerable evidence that the embryos of higher plants have a metabolism more directly comparable to that of animals and micro-organisms, than is the metabolism of differentiated stem and leaf structures. For example, Merry and Goddard (1941) and Marsh and Goddard (1939) have found that embryos and roots of barley and carrots have a respiration sensitive to HCN, NaN_3 and photoreversibly with CO, and the respiration is therefore presumably

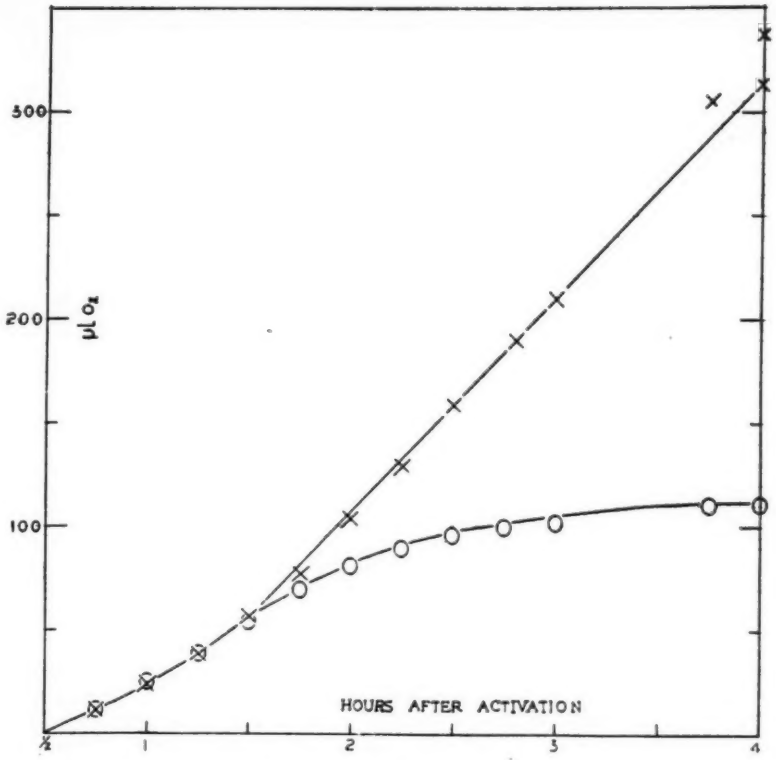


FIG. 6
OXYGEN CONSUMPTION OF ACTIVATED *Neurospora tetrasperma* ASCOSPORES AT 25° C; CROSSES, CONTROLS, CIRCLES 0.001 M IODOACETAMIDE ADDED BEFORE HEAT ACTIVATION. GODDARD UNPUBLISHED.

catalyzed by a cytochrome oxidase—cytochrome c system. The mature leaves of these plants, including wheat (Allen and Goddard, 1938) are not inhibited by these same poisons. These results are illustrated in figs. 7, 8 & 9. In the case of wheat, both the presence of cytochrome oxidase and cytochrome c has been demonstrated (Brown and Goddard, 1941; Goddard, 1944). In differentiation of the leaves there has been a change in metabolism that either blocks the sensitivity of the cytochrome oxidase to these poisons, or a new oxidase system has been developed which replaces the cytochrome oxidase.

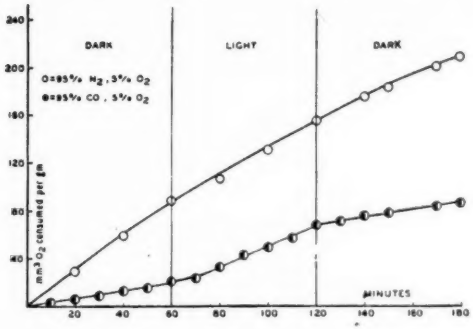


FIG. 7

CO Inhibition of Wheat Embryos and Its Reversal by Light. From Brown and Goddard (1941).

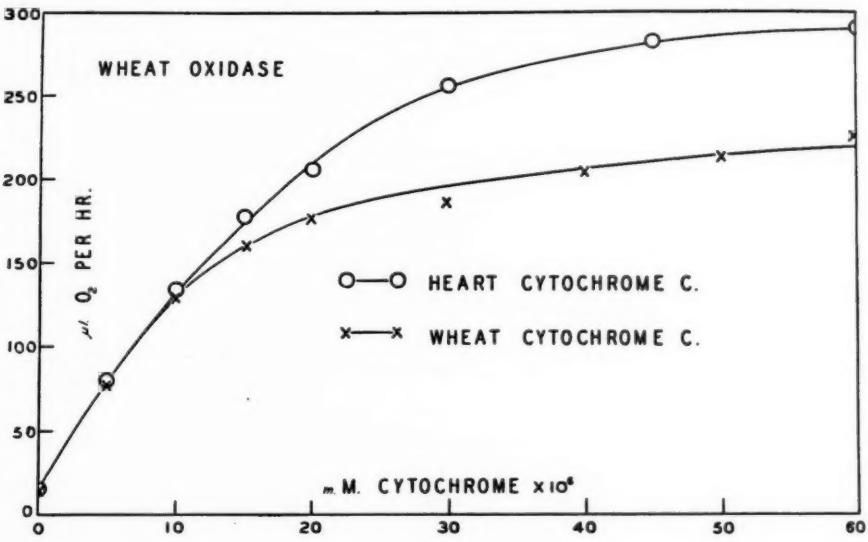


FIG. 8.

The Activity of Cytochrome Oxidase from Wheat Embryos with Cytochrome C Prepared from Heart and Wheat; Substrate Hydroquinine, pH 7-1, t = 25 C. From Goddard (1944).

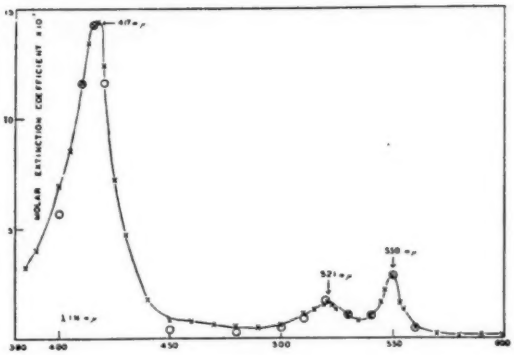


FIG. 9

THE ABSORPTION SPECTRUM OF CYTOCHROME C ISOLATED FROM WHEAT. CIRCLES, HEART CYTOCHROME C. FROM GODDARD (1944).

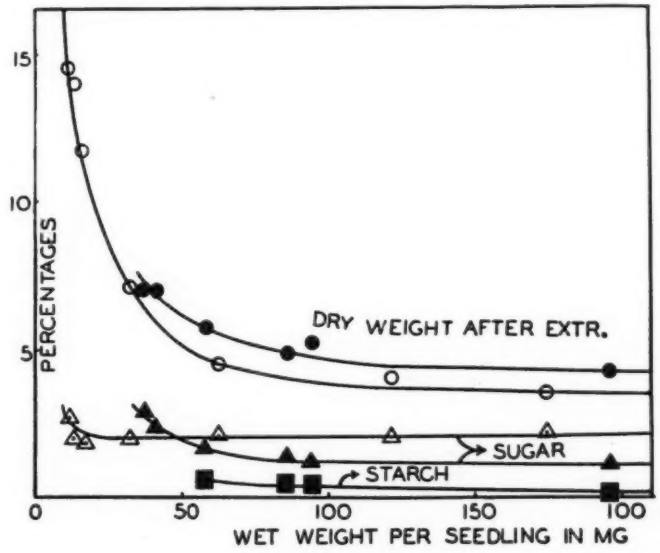


FIG. 10

CHANGES IN DRY WEIGHT AND CARBOHYDRATE IN PEA SEEDLINGS (MINUS COTYLEDONS) IN RELATION TO AGE. UNPUBLISHED EXPERIMENTS OF DR. B. J. D. MEEUSE.

A seed is a dormant structure containing a plant embryo in a state of diapause. Usually water and air are among the essential conditions to initiate its development; however, in the early phase of the growth of the embryo, conditions are partially anaerobic, with a considerable evolution of CO_2 of fermentation resulting in a high R.Q. as is shown in fig. 1. Dr. Jean Phillips (1947) working in my laboratory has shown that young rice seedlings have a high fermentative capacity with a pure alcoholic fermentation with a ratio of alcohol/CO_2 of unity. As the seedling becomes older the fermentative capacity decreases and the ratio falls below unity, as is shown in Table I.

TABLE I

FERMENTATION IN RICE SEEDLINGS

Hrs. in N_2	mM. CO_2	delta mM. alc.	CO_2/alc.
	A—Before 1st leaf emerges		
6	1.45	1.42	0.98
9	0.49	0.52	1.06
23	5.20	5.11	0.98
23	5.46	5.44	1.00
	B—After 1st leaf emerges		
6	2.33	1.20	0.54
6	1.80	1.37	0.76
6	1.47	1.32	0.90
12	0.52	0.37	0.71

Barley seedlings from the first produce more CO_2 than alcohol, and the extra CO_2 comes from displacement of preformed CO_2 in the seedlings. The most interesting feature of these results was the complete absence of anaerobic alcoholic fermentation from the mature barley leaves. These leaves always contain some alcohol, but there is no increase in alcohol during an anaerobic period.

Perhaps even more striking results have been obtained in my laboratory this year by Dr. B. J. D. Meeuse. The young pea seedlings have a fairly low respiration which increases with growth, reaching a maximum level at 4 days, as may be seen from fig. 11. The young seedlings have a pure alcoholic fermentation, of fairly high capacity. Meeuse (1948) has represented this as I/N ratio, which is the ratio of CO_2 produced anaerobically to CO_2 aerobically. His results are represented in fig. 12 and it may be seen that the ratio falls from

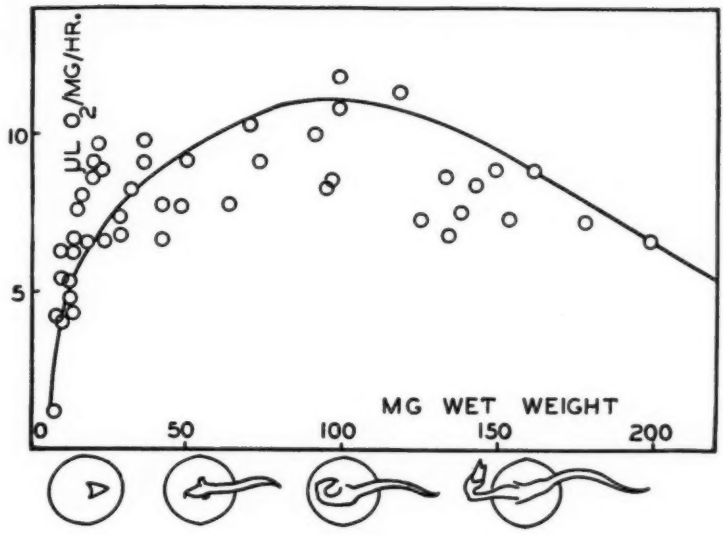


FIG. 11
RESPIRATORY RATE OF PEA EMBRYOS (MINUS COTYLEDONS) AGAINST INCREASING AGE (WEIGHT) OF SEEDLINGS. UNPUBLISHED RESULTS OF DR. B. J. D. MEEUSE.

slightly over unity to about 0.4. From the equations of respiration and of fermentation:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

and

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

it may be readily seen that when the I/N ratio is 1.0, glucose is broken down three times as fast anaerobically as aerobically. If the ratio is 0.33 the rates are identical.

There is a considerable body of respirometric work which indicates a Pasteur effect, that is a lower rate of glucose utilization aerobically than anaerobically in plant tissues, but surprisingly, there is no analytical evidence in the literature that actually proves this point. Meeuse, on a basis of carbohydrate balances, has shown conclusively that young pea embryos have a good Pasteur effect. Only a summary of his evidence is presented here, in fig. 13, where he has plotted the ratio of carbohydrate disappearance anaerobically over the carbo-

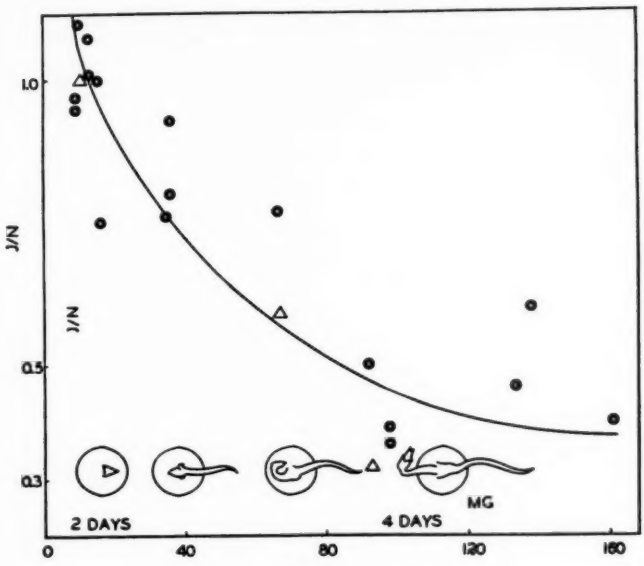


FIG. 12
RATIO OF ANAEROBIC CO_2 TO AEROBIC CO_2 OF PEA EMBRYOS (MINUS COTYLEDONS) OF INCREASING AGE. UNPUBLISHED RESULTS OF DR. B. J. D. MEEUSE.

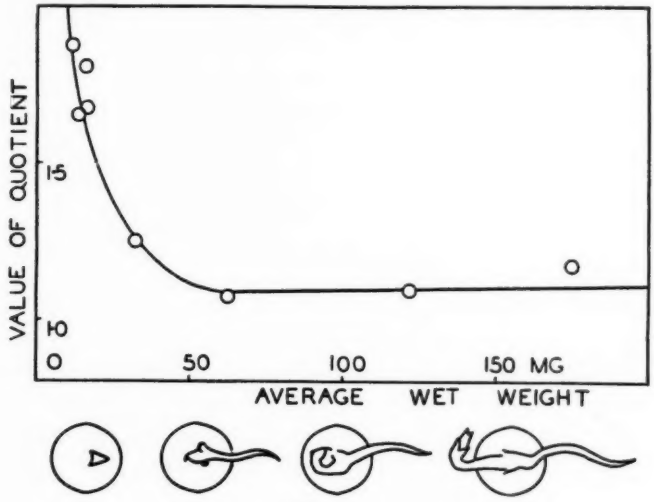


FIG. 13
THE QUOTIENT OF CARBOHYDRATE DISAPPEARING IN NITROGEN TO THAT DISAPPEARING IN AIR FOR PEA EMBRYOS (MINUS COTYLEDONS). FROM UNPUBLISHED RESULTS OF DR. B. J. D. MEEUSE.

hydrate disappearance aerobically. It may be observed that as the seedlings develop, the Pasteur effect decreases markedly, and with a ratio of unity no Pasteur effect is demonstrated. From the I/N values, the expected ratio is 3.0, but this is on the assumption that all the carbohydrate that disappeared was fermented or respired. Under anaerobic conditions, there is no growth, and this assumption is probably justified, but in the presence of air there is considerable growth during the experimental period and a considerable fraction of the carbohydrate is converted to cellulose, proteins, etc. From the I/N ratio and the actual values of the anaerobic/aerobic carbohydrate disappearance, one may calculate the amount of carbohydrate used in respiration and the amount in growth; the calculation indicates 60% is respired and 40% used in synthetic reactions; and this checks well with the non-carbohydrate increase in dry weight.

Though fermentation under anaerobic conditions is well known in higher plants, there appears to be a marked diminution in its metabolic role in the growth of seedlings, and it is at a maximum during the early stages of germination where a portion of the energy for growth would appear to have a fermentation origin.

The utilization of organic food in growth is a problem of many aspects. First, we may consider the amount of the food that is converted into new material. Some results are presented in Table II.

TABLE II.
GAINS IN BODY WEIGHT PER KCAL. FOOD

Organism	gm. dry wt.	Reference
Pig	.036—.073	Armsby & Moulton from Rahn (1940)
Trout	.068	McCay from Rahn (1940)
Cockroach	.081—.083	McCay from Rahn (1940)
Yeast	.135	Classen from Rahn (1940)
Trichophyton (a fungus)	.131	Calc. from Goddard (1934)
Barley (seedlings)	.179	Calc. from Barnell (1937)
Aspergillus niger	.125—.145	Terroine from Rahn (1940)
Bacillus coli	.075—0.181	Rahn (1940)

One difficulty with all such data is that the time periods of the growth vary enormously, and therefore the periods during which part of the food must be used for maintenance. For example, the growth period over which these studies were made varied from a few hours in bacteria to months in pigs. Since the amount of growth resulting from a kcal. of food are not greatly different, one might, as a first approxi-

mation, suggest that the energy of maintenance is essentially a constant fraction of the food over the whole growth period, rather than its utilization at a constant rate.

If one asks what the energy liberated in respiration is used for in growth, some real difficulties are apparent. One might suggest at least three processes; osmotic work, increase in surface, and synthesis of new compounds. Some preliminary efforts will be made to deal with these problems.

Let us consider the osmotic work done by a barley root cell in doubling its volume from 8000 μ^3 to 16,000 μ^3. Such a cell has an internal osmotic pressure of approximately 5 atmos. or the equivalent of a 1.22 M solution, and it will grow readily in a solution of 0.05 atmos. equivalent to a 0.0122 M solution. We will assume that as it doubles in volume it does not lower its osmotic pressure, and that the osmotic pressure is maintained entirely by active absorption from the solution. The osmotic work to move n. molecules from the lower conc. A_1 to the higher concentration A_2, is then defined by the equation:

$$\Delta F = nRT\ 1n \frac{A_2}{A_1} = n2.303\ RT \log \frac{A_2}{A_1}$$

where ΔF = free energy increase
R = 1.987 cal. per degree per mole
A_2 = molar conc.
A_1 = molar conc.
n = no. moles removed
T = absolute temperature

For a cell to change from 8000 μ^3 to 16,000 μ^3 without change in osmotic pressure, the osmotic work is 26×10^{-3} μcals., where *a* μcal. is 10^{-6} cals. The respiratory rate of dividing barley root cells, is from Machlis (1944) results 45 μl. per μl. of tissue per hour, with an R.Q. of nearly unity. This represents an O_2 consumption of 5.4×10^{-4} μl O_2 per cell per hour since at R.Q. of 1.0, 1 μl $O_2 = 4.825 \times 10^{+3}$ μcal., this furnishes the cell with 2.6 μcal., per hour. Thus, if we assume 100% efficiency in osmotic work, 1% of the respiratory energy could bring about a doubling of the cell volume in 1 hour, while if the efficiency was as low as 5%, then 20% of the respiratory energy would be required. Similar calculations can be made for *Escherichia coli,* with the same assumptions about internal and external osmotic pressures

that were made for barley, and a generation time of 40 minutes. Here the osmotic work represents but 0.25% of the total work.

As we shall see later, the living cell is probably a molecular engine, and the real relationships are correspondence between molecules reacting in downhill reaction and molecules synthesized. The recent results of Davies (1948) and Robertson (1948) seem to suggest that four ions may be transported across the cell surface for each extra O_2 absorbed, this is of interest since each O_2 accepts 4 electrons in respiration. If this is true the calculated thermodynamic efficiencies have little meaning. It is interesting to note that for the barley root cells about 5.8×10^{14} molecules of solute are absorbed in doubling its volume, and such a cell absorbs 1.46×10^{16} O_2 molecules per hour or 5.8×10^{16} electrons are available for use in ion transport.

It is sometimes stated that the increase in surface energy in cellular growth requires a considerable expenditure of energy. The increase in surface area results in an increase in surface energy and this may be represented as follows:

$$\Delta F = \lambda \text{ ergs cm.}^2 \text{ per cm. increase.}$$
$$\text{where } \lambda = \text{surface tension in dynes per cm.}$$

If an E. coli cell doubles in volume from 0.75 μ^3 to 1.50 μ^3 its surface area increases from 3.98 μ^2 to 4.44 μ^2. Converting to calories, we find that each μ^2 increases in surface at 1 dyne per cm. surface tension is equal to 2.4×10^{-10} μcals. The surface tension of bacteria may not be known, but if we assume it is low, say 2 dynes, as in some cells investigated by Danellei and Harvey (1934), the increase in surface energy in doubling its volume is 1.1×10^{-9} μcal., while its respiration furnishes 1×10^{-3} μcals. which is 10^6 times as large. Even a surface tension 100 times as great, would require but a small portion of the energy available.

Synthesis of Organic Compound

One of the fundamental aspects of growth is the formation of new protoplasm, that is the synthesis of all of the organic compounds that make up a particular protoplasm and their arrangement in the specific submicroscope and microscope organization that results in a functioning living system.

The organic food must serve not only as the structural materials

for new synthesis but furnish any required energy for the uphill steps in the synthetic reactions, and to supply the required energy for the orderly and specific arrangement of the parts.

In the synthesis of proteins, it is not enough to synthesize new peptid bonds, but the arrangement of the particular pattern of the amino acids must be duplicated, and the polypeptid chains must be arranged and folded in such a manner as to reproduce the highly ordered structure of a specific globular or fibrous protein. It has been proposed by Emerson (1945) that these syntheses may occur on the gene surfaces or on surfaces which are mirror images of the gene. Casperson (1948) has proposed that nucleoproteins are essential to protein synthesis.

In many cases the particular substances produced are formed in spontaneous reactions, that is in enzymatically catalyzed reactions which proceed with a decrease in free energy. For example the formation of lactic and pyruvic acids from carbohydrates, and the various keto acids and aldehydes which occur in the tricarboxyllic acid cycle. In such reactions, which we may illustrate with pyruvic acid:

$$C_6H_{12}O_6 + 2 \text{ coen.} \rightleftharpoons 2 \begin{array}{l} CH_3 \\ | \\ C = O \\ | \\ COOH \end{array} + 2 \text{ coen. } H_2$$

$$\Delta F = -48.1 \text{ kcal.}$$

and the equilibrium lies far toward the side of pyruvic acid. The free energy decreased is described by the relation:

$$\Delta F = \Delta H - T\Delta S$$

where ΔH = change in heat content in kcals/mole
ΔF = change in free energy in kcals/mole
T = absolute temperature
ΔS = change in entropy in kcals. per degree

The change in free energy is the particularly interesting term, because it determines the equilibrium constant:

$$\Delta F = -RT \ln K = -2.303 \ RT \log K$$

where R = 0.001987 kcals. per degree per mole
K = equilibrium constant

If ΔF is zero, $K = 1$, and if ΔF is negative K is larger than one and the equilibrium is such that the reaction proceeds from left to right. If ΔF is positive, the equilibrium constant is less than unity, and at equilibrium, the concentration of the products on the left are higher than the concentration on the right. This is illustrated by Table III.

TABLE III
EQUILIBRIUM CONSTANT AND FREE ENERGY OF REACTION

K	ΔF in kcals.	% reaction for equilibrium
10,000	—5.480	99.99
1,000	—4.010	99.9
100	—2.740	99
10	—1.370	90
1.0	0	50
0.1	+1.370	10
0.01	+2.740	1
0.001	+4.010	0.1
0.0001	+5.480	0.01

Where:

$$A \rightleftharpoons B;$$

$$\frac{B}{A} = K$$

We may see that at 25° no appreciable reaction will occur where ΔF is positive to more than a few kcals. per mole.

Yet living cells have the unique capacity of carrying out innumerable reactions of large positive ΔF. We may illustrate this with the reduction of nitrate to the equivalent of NH_3, a process that occurs in many plants:

$$HNO_3 + H_2O \rightarrow NH_3 + 2O_2;\ \Delta F = 84 \text{ kcal.}$$

The ΔF is so large, that the equilibrium concentration of NH_3 has no meaning, for at 0.1 M HNO_3 and O_2 at atmospheric pressure, the equilibrium concentration of NH_3 would be about 3.4×10^{-61} M. We realize that this reaction does occur, and is presumably coupled with carbohydrate respiration:

$$C_6HO_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O;\ \Delta F = -686 \text{ kcal.}$$

However, the mechanism of energetic coupling is still unsolved.

It is not possible to calculate the work which is done in synthesizing the substances required in growth from a knowledge of the heat con-

tent of the diet and the heat content of the organism, since such a calculation would have to assume that in all of the reactions $\Delta S =$ zero, and $\Delta F = \Delta H$. In many of the reactions, ΔS may be small, and the difference between ΔF and ΔH not great. However, when one considers the high degree of organization of proteins, the entropy term may not be small, and the free energy increase of synthesis from the amino acids may be considerably higher than that calculated from the sum of the peptid bond synthesis. We have no measure of the entropy decrease in protein synthesis, but it is interesting to note that in protein denaturation the free energy of activation is very much lower than the heat of activation. The values calculated by Eyring and Stearn (1939) are: $\Delta F^{\ddagger} = 25.4$; $\Delta H^{\ddagger} = 132.1$ and $T\Delta S^{\ddagger} = 166.7$. These values are of no direct application to problem of protein synthesis, but they do demonstrate that large entropy changes may be associated with protein molecules.

In general, we might point out that a reaction of positive ΔF may be driven, if it is energetically coupled with a reaction which has a sufficiently large free energy decrease. We might illustrate this as follows:

$$\begin{aligned} A &\rightarrow B \\ &\quad \Delta F_1 = +x \\ C &\rightarrow D \\ &\quad \Delta F_2 = -Y \end{aligned}$$

If coupling occurs:

$$\begin{aligned} &A + C \rightleftharpoons B + D \\ &\Delta F_3 = \Delta F_1 - \Delta F_2 = X - Y \\ &\text{If } \Delta F_3 < O, \end{aligned}$$

the equilibrium should be toward B + D. One will expect the greatest thermodynamic efficiency when $\Delta F_1 = -\Delta F_2$; however, if $\Delta F_2 < -\Delta F_1$, one will expect a considerable portion of the energy to be wasted. Even if the free energy decrease of the driving reaction is somewhat less than the free energy increase of the driven reaction, it is theoretically possible for the reaction to proceed if there is a sufficient excess in concentration of the energy rich compound. For example, in the above reaction, if the concentration of C is 100 times the concentrations of A, B, and D, the reaction will proceed to convert ½ of

A to B, if ΔF_1 is 2.7 kcal. smaller than $-\Delta F_2$. In this particular case the efficiency will be more than 100%, if one considers only the two reactions, and the difference in energy will have to be made up by the absorption of heat from the surroundings.

Thermodynamic considerations will never show us what reactions actually occur, at what velocity, nor the pathways followed. It is useful in interpretation, in showing what are the possibilities, and in eliminating the impossible reactions.

There are only a few cases where much is known considering the details of energetic coupling. Oxidative-reductive coupling is possible where two systems of different potential may be mediated by an electron mobile system, reacting with each system. For example, Borsook and Schott (1931) have shown that pyruvate may be reduced to lactate by energy furnished by the oxidation of formate. However, catalysts for each partial reaction were required; they used toluene treated *Bacillus coli,* and an electron mobile substance such as janus green or methylene violet. The reactions could be represented as follows:

pyruvate ion + 2H + + 2e = lactate ion
$E'_0 = +0.180$ volt (pH 7.0)
$\Delta F_1 = +8.30$ kcal.
formate + H_2O = HCO_3 + 2H + + 2e
$E'_0 = -0.41$ volt (pH 7.0)
$\Delta F_2 = -18.90$ kcal.
pyruvate ion + formate + H_2O = lactate ion + HCO_3—
$\Delta F_3 = \Delta F_1 + \Delta F_2 = -10.60$ kcal.

Similarly Green, Devan and Leloir (1937) showed that the reduction of several substances could be coupled to the oxidation of β-hydroxybutyrate using coenzyme I as an electron carried.

The role of phosphate in metabolism and in energy transfer is a large one and has been reviewed elsewhere (Lipmann, 1941, 1946; Kalchar 1941). Here we will be concerned with but one aspect of the problem, the well established fact that energy released in oxidation of carbohydrates may be coupled to the synthesis of organic phosphorus compounds and that this coupled energy may be used later for various synthetic reactions.

It had long been recognized that phosphate was an essential constituent of cellular metabolism. Lohmann's (1931) discovery of

adenosine triphosphate (A.T.P.) opened up a new phase of the problem. Meyerhof, Ohlmeyer and Mohle (1938) made the illuminating discovery that the oxidation of phosphoglyceraldehyde was coupled to phosphorylation of adenosine diphosphate. Warburg and Christian (1939) and Negelein and Bromel, worked out the actual steps involved. These reactions can be illustrated as follows:

(1) $CH_2OPO_3H_2$ | CHOH | CHO $+$ coen. $+ H_2O \rightleftharpoons$ $CH_2OPO_3H_2$ | CHOH $+$ coen. H_2 | COOH

$\Delta F = -16.5$ kg. cal.

(2) A.D.P. $+ H_3PO_4 \rightleftharpoons$ A.T.P.

$AF = 11\text{-}14$ kg. cal.

Now reaction (1) will not proceed without the presence of phosphate, there is a compulsory coupling of phosphorylation to oxidation, and the reaction proceeds as below:

$CH_2OPO_3H_2$ | CNOH | CHO $+ H_3PO_4 +$ coen. $\rightleftharpoons$ $CH_2OP_3H_2$ | CHOH $+$ coen. H_2 | C—OPO_3H_2 | OH

$CH_2OPO_3H_2$ | CHOH | $COPO_3H_2$ | OH $+$ A.D.P. $\rightleftharpoons$ $CH_2OPO_3H_2$ | CHOH | COOH $+$ A.T.P.

This was the first reaction known in which we had definite information of the coupling of oxidative energy into a particular synthetic reaction. It is also known that phosphopyruvic acid can transfer a phosphate to A.D.P. and convert it to A.T.P. with the formation of pyruvic acid. Since two phosphoglyceraldehyde molecules and two of

phosphopyruvic acid are formed in the fermentation of one glucose, 4 A.T.P. molecules are synthesized from A.D.P. per glucose fermented. Since two A.T.P. are used up in the conversion of glucose to fructose diphosphate, there is a net gain of two A.T.P. We might summarize these reactions as follows:

$$\text{glucose} \rightleftharpoons 2 \text{ alcohol} + 2CO_2$$
$$\text{or } \Delta F = -54 \text{ kg. cal.}$$
$$\text{glucose} \rightleftharpoons 2 \text{ lactate}$$
$$\Delta_1 F = -54 \text{ kg. cal.}$$
$$2 \text{ A.D.P.} + 2H_3PO_4 \rightleftharpoons 2 \text{ A.T.P.}$$
$$\Delta_2 F = +22 \text{ to } 28 \text{ kg. cal.}$$
$$\text{glucose} + 2H_3PO_4 + 2 \text{ A.D.P.} \rightleftharpoons 2 \text{ alcohol} + 2CO_2 + 2 \text{ A.T.P.}$$
$$\Delta F_2 = -32 \text{ to } -26 \text{ kg. cal.}$$

Ochoa (1943) has shown that in the respiration of glucose 36 A.T.P. molecules are synthesized, or recalling that two are used, a net gain of 34. Since the oxidation of glucose releases approximately 700 kg. cal., and the 34 A.T.P. molecules represent a gain of about 408 to 476, we see that between 58 and 68% of the energy released in respiration may be stored in the synthesized A.T.P.

If the energy remained locked as the potential energy of adenosine triphosphate (the so-called phosphate bond energy) no useful purpose would be served by cellular metabolism. The interesting point is that A.T.P. may undergo a wide variety of enzymatically catalyzed chemical reactions in which phosphate is transferred to other molecules and in which the molecules gaining the phosphate also gain some or all of the potential energy difference between A.T.P. and A.D.P. These reactions will serve to regenerate A.D.P., so that even though the quantity of A.D.P. and A.T.P. in living tissues is low, it may serve, in a cyclical manner, to transfer large amounts of the energy released in cellular metabolism.

We cannot consider the wide range of reactions in which A.T.P. participates. but will consider the synthesis of polysaccharides from glucose. Polysaccharides may be readily hydrolyzed by diastastic enzymes to glucose, as follows:

$$\text{Starch or glucogen} + (n\text{-}1)\ H_2O = n \text{ glucose}$$
$$\Delta F = -1 \text{ to } -3 \text{ kg. cal. per mole of glucose.}$$

This reaction has never been reversed in the test tube, and even though the increase in free energy per mole of glucose is small, the equilibrium lies far to the right. However, a different pathway has been found to be effective in living cells. Glucose may be phosphorylated by A.T.P. in the presence of the enzyme hexokinase:

$$\text{glucose} + \text{A.T.P.} \xrightarrow{\text{hexokinase}} \text{glucose-6-PO}_3\text{H}_2 + \text{A.D.P.}$$

$$\Delta F = -8 \text{ kg. cal.}$$

Some of the energy gained by synthesis of A.T.P. is stored as potential energy of the glucose-6-PO_3H_2. The glucose-6-PO_3H_2 may now be converted to glucose-1-PO_3H_2 by the enzyme phosphoglucomutase.

Lastly, as demonstrated in the brilliant work of C. F. and G. T. Cori (1938; 1945) glucose-1-PO_3H_2 may be converted to polysaccharide in the presence of the enzyme, phosphorylase:

$$\text{n glucose-1-PO}_2\text{H}_3 \xrightleftharpoons{\text{phosphorylase}} \text{polysaccharide} + \text{n } \text{H}_3\text{PO}_4$$

This reaction requires a trace of polysaccharide for its initiation, and the equilibrium will depend upon the concentration of H_3PO_4, pH, temperature, and the concentration of glucose-1-PO_3H_2.

It is interesting to note that one A.T.P. is required for each glucosidic linkage synthesized in the polysaccharide, in other words there is a one to one correspondence, between molecules furnishing and molecules accepting the energy. In this case the efficiency is fairly low, of the order of 25% or less. It is also of interest to recognize that the complete oxidation of one glucose molecule can result in the conversion of 34 molecules of glucose to polysaccharide.

There is some reason to believe that A.T.P. may furnish the energy for the synthesis of proteins from amino acids (Cohen and McGilvery, 1946, 1947).

Our interest in the problem here is a general one, and not in biochemical details, which could be more adequately discussed by someone else.

The particularly interesting conclusion is, that the enzymes serve not only to speed up tremendously the rate of the chemical reactions,

but serve to transfer energy from one chemical reaction which proceeds with a free energy decrease to another reaction which proceeds with a free energy increase. It is interesting also to note that these reactions, and the few other coupling reactions which are known, occur under such conditions that there is a one to one correspondence between molecular reactions furnishing and accepting the energy.

Two possible conclusions may be drawn from this: (1) that the energy transfer probably occurs in a trimolecular molecular complex with the enzyme. Such molecular complexes have already been postulated on kinetic grounds, and there is an abundance of indirect evidence that in oxidation reduction reactions a tricomplex exists between electron donor and electron acceptor. The literature bearing on this problem has been reviewed elsewhere by LuValle and Goddard (1948). (2) That efforts to calculate thermodynamic efficiencies of synthetic reactions will have little meaning, since one molecule will be synthesized from one degraded, and the energy stored may vary from approximately 100% to zero.

In other words, the metabolic engine is presumably a molecular engine, and thermodynamic considerations will allow us to choose from postulated mechanisms those which are possible and those which are impossible, but only the detailed knowledge of the individual stepwise reactions, as they are unfolded by the metabolic biochemist, or by specific blocking by vitamin, amino acid, and hormone analogs, or by knowledge obtained from gene controlled reactions, will complete the picture of the master chemist, the living cell; and the means by which it resists the drift towards equilibrium, reverses the direction and proceeds to grow, to differentiate, and to reproduce.

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GROWTH AND ANTERIOR PITUITARY

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It has been known for many years from clinical observations that the pituitary gland exerts a direct control on the body growth and size. Early experiments of Smith (1916) and Allen (1916) on tadpoles, and of Crowe, Cushing and Homans (1910) on dogs have confirmed these observations. From the classical studies of Smith (1926, 1930), it is clear that following hypophysectomy, there is (1) no further increase in general body weight, (2) inhibition of skeleton growth, (3) regression in weights of the adrenal cortex, the thyroids and the reproductive organs, and (4) decrease in size of the liver, spleen and kidneys. Conversely, daily anterior homeo-transplants or injections of anterior pituitary extracts restore the hypophysectomized animals to a normal or nearly normal condition. Thus, there are two types of growth-stimulating substances in the anterior lobe of hypophysis: one concerns *specific* organ growth and the other *general* body growth. Although the specific growth substances stimulate only the development of the specific organ (for instance, adrenocorticotropic hormone stimulates the enlargement and function of the adrenal cortex), they often affect indirectly the process of general body growth.

There seems to be little doubt that we can separate and purify six hormones from the anterior pituitary extracts. Four (lactogenic, adrenocorticotropic (ACTH), interstitial-cell stimulating (ICSH) and growth hormone) of these six hormones have been isolated in pure state. Experiments using these pure hormones in recent years have much clarified our biological knowledge of the anterior pituitary. From these studies, we have found that adrenocorticotropic and thyrotropic hormones directly or indirectly influence the growth-promoting action

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of the growth hormone. In the following, I will attempt to discuss in some detail this important subject of the interplay between these hormones.

It may be well at this moment to summarize our present knowledge in the chemistry of anterior pituitary hormones (reviewed in Li, 1947). It is now well established that these hormones are proteins: some (FSH, ICSH and thyrotropin) contain carbohydrate and others do not. Follicle-stimulating hormone (FSH) is characterized by its solubility in 2.0*M* $(NH_4)_2\ SO_4$, lactogenic and growth hormones by their insolubilities in water. Biological activity of these hormones may be destroyed by acetylation and iodination indicating that both primary amino and free tyrosine groups are essential for their intrinsic hormonal functions. Adrenocorticotropic hormone is exceptionally stable toward peptic digestions and heat, whereas other hormones are inactivated by such treatments. This indicates that certain peptide fragments may possess adrenocorticotropic potency.

The same hormone isolated from the pituitary gland of one species may not be identical chemically to that obtained from other species. For instance, interstitial-cell stimulating hormone isolated from pig glands has a molecule weight of 90,000, while the same hormone obtained from sheep, 40,000, although their biological behavior cannot be differentiated.

There is no evidence at the present to indicate that anterior pituitary hormones possess prosthetic group(s). Analytical data does not detect any peculiarity in the amino acid components of these protein hormones. The amino acid composition of the growth hormone, for example, as determined by microbiological and colorimetric methods reveal that the amount of each amino acid in the hormone is within the normal values found in other proteins.

I. Specific Growth Substances (the Tropic Hormones)

The pituitary tropic hormones (lactogenic, thyrotropic, gonadotropic, and adrenocorticotropic hormones) may be called *specific* growth substances, for their function is to enlarge the size of an endocrine organ and, in turn to enhance the activity of that gland:

Lactogenic hormone ..... Mammary gland
(crop gland in pigeons)

Thyrotropic hormone ..... thyroids

Gonadotropic hormones ..... ovaries in females and testes in males.

If we take the weight gain as one of the criteria of growth, these tropic hormones are powerful growth-stimulating agents. Let us examine the action of gonadotropic hormones on the ovaries of immature female rats. It is well established that the growth of the follicles in the ovary depends upon the secretion of FSH from the pituitary, and their growth to maturity, on the other hand, is controlled by the action of ICSH. The size of the ovary could be made to increase considerably when a combination of these two gonadotropic hormones is administered into the animal. This increment of ovarian size is mainly due to the stimulation of FSH on the cells of the follicles by intense mitotic division and the hypertrophy of the interstitial-cells caused by ICSH.

The work of Riddle, Lyons, Turner and their collaborators have shown that lactogenic hormone has a direct action on the development of mammary glands; under certain conditions, the lactogenic hormone actually induces milk formation. Thus, we have a hormone which concerns the synthesis of milk protein in addition to its function for the growth of mammary gland. There is another anterior pituitary hormone which stimulates the gland to synthesize a hormonal protein and which is also responsible for the growth of the gland. This hormone is called thyrotropic hormone and the protein it catalyses to form is thyroglobulin in the thyroids. Recent experiments of Chaikoff and his co-workers have clearly shown that a thyrotropic hormone enhances the uptake of iodine in the gland for the synthesis of the thyroid hormone using radioactive iodine as the tracer.

The size and development of adrenal cortex are under the control of pituitary adrenocorticotropic hormone. The adrenal cortical hormones, which are formed by the stimulation of ACTH, have definite influence to the general body growth. This subject will be discussed later. It is sufficient to say that one of the biological characteristics of ACTH is to inhibit the growth of the whole animal.

II. Body Growth

The pioneer work of Evans and Long in 1921 has shown that true gigantism could be produced in rats by the injection of anterior pituitary extracts. Later Putnam, Benedict and Teel (1929) using similar extracts in dogs produced not only an increased growth rate but also some characteristic features of clinical acromegaly. The existence of a growth-promoting substance in the anterior pituitary is further supported by the work of Smith (1926, 1930) who found that the body growth of hypophysectomized rats can be resumed by the daily implants of the anterior lobe of hypophysis. However, there has been some doubt as to the ability of the growth hormone to cause *continuous* growth in normal and in hypophysectomized rats. Recent experiments with normal adult female rats, after the administration of pure hormone for 435 days, indicate that growth continued during the whole period with no sign of a lack of responsiveness (Evans, Simpson and Li, 1948). A similar experiment with hypophysectomized female rats also showed that there is no indication of refractoriness after 437 days of injections. It must be emphasized here that other endocrine glands in these rats did not show any sign of stimulation as judged by their size and histological examination. It is significant that the growth-promoting action of the hormone does not require the internal secretions of adrenal cortex, thyroid, pancreas, and gonads. This is also evident from earlier experiments using crude pituitary extracts in adrenalectomized, thyroidectomized, pancreatectomized or gonadectomized animals. (See review by Long, 1943.) However, the biological potency of the growth hormone may be modified by the functional state of these endocrine glands.

Smith in 1933 has shown that the thyroid hormone exerts a synergistic effect on the growth activity of anterior pituitary extracts. This was later confirmed and extended by Evans, Simpson and Pencharz (1939). Therefore, it is not surprising to find that thyrotropic hormone enhanced the capacity of growth hormone to stimulate body weight gain in the hypophysectomized rat (Marx, Simpson and Evans, 1942). A typical experiment of the synergism in body growth between the pure growth hormone and thyroxin is that of Becks, Simpson, et al. (1946) in which they found that the thyroid hormone not only augments the body-weight increasing activity of growth hormone but also the skeleton changes.

There are few reports in literature relating the direct influence of thyroid hormone to body weight gain of the animal. Dott (1923) observed that feeding small amounts of thyroid tissue to immature dogs always resulted in acceleration of the growth rate. In rats, Schafer, as early as 1912, reported that the rate of growth and feed intake of female rats were greatly enhanced by feeding small amounts of thyroid. Increased rates of growth of mice as the result of thyroid feeding was also found by Robertson (1928). Koger, Hurst and Turner (1942) confirmed Robertson's observation and found that the thyroxin-injected mice gained more body weight per unit of food intake than did the controls. The fact that small amounts of thyroid hormone causes an increase of body growth in normal animals may be supported by an indirect evidence that mild hyperthyroidism is conducive to rapid growth. Turner and his co-workers (1940, 1941) also found that the content of thyrotropic hormone in the anterior pituitary is higher during the period of rapid growth of rats and rabbits.

The influence of hyperadrenocortism, as produced by the injection of adrenocorticotropic hormone, on somatic growth has been described by Evans, Simpson and Li (1943). When young growing male rats (26 days of age) were treated with adrenocorticotropic hormone for 30 days, it was found that the treated animals gained only 80 gm whereas the uninjected controls 154 gm. This inhibiting effect of ACTH on body weight gain is in harmony with earlier observations of Ingle, Higgins and Kendall (1938) where adrenal cortical hormones were used. It was further shown that the action of ACTH counteracts the growth-promoting activity in hypophysectomized rats when these two hormones are administered simultaneously. This antagonism of ACTH to growth is also observed in studies of skeleton growth (Summarized by Li and Evans, 1947).

III. Urinary Nitrogen

Since true growth is generally interpreted as the accumulation of proteins, it is reasonable to expect, from the foregoing discussion, that an important function of the growth hormone should be to retain nitrogen for protein synthesis. Recent studies with the pure growth hormone have shown that the hormone reduces urinary nitrogen markedly under various conditions. In some cases, the disappear-

ance of urinary nitrogen corresponds almost quantitatively to the gain in body weight. The hormone causes an evident decrease of urinary nitrogen excretion during the first 24 hours and the decrease persists throughout the injection period. After cessation of hormone treatment, the urinary nitrogen returns within a day to the pre-injection level. From the value of nitrogen retained, the amount of tissue (protein), which may be added to the animal body, can be calculated by assuming that the tissue formed has 20 per cent protein and that the protein contains 16 per cent nitrogen. Computed value as obtained by Gordon, Bennett, et al. (1948) was found to be in fair agreement with the observed increment in body weight. Thus, the nitrogen retention, as a result of growth hormone treatment, is sufficiently accounted for by the gain in body weight which indicates the formation of new tissues. Similar experiments using hypophysectomized rats reach the same conclusions.

The nitrogen-retaining effect of the growth hormone has also been demonstrated in rats with bilateral fracture of the femur (Bennett, Applegarth and Li, 1946) and in rats made diabetic by alloxan (Bennett and Li, 1947).

If the lowering of nitrogen excretion is one of the intrinsic properties of the growth hormone, adrenocorticotropic hormone may very likely produce an increase in urinary nitrogen. The study of Ingle, et al. (1946) in normal rats force-fed a high carbohydrate diet showed that the administration of ACTH caused an increase in the excretion of nitrogen and potassium and inhibition of growth. An enhancement of nitrogen excretion in alloxan-diabetic rats after ACTH injection has also been observed by Bennett and Li (1947). Clinical investigations using ACTH give identical results (Mason, Powers, et al., 1948; Forsham, Thor, et al., 1948). The increase in urinary potassium during the administration of ACTH probably resulted from the increased breakdown of body tissues.

IV. Body Composition

From the foregoing discussion, it may be assumed that the nitrogen-retaining effect of the growth hormone is due to the result of the catalysis in the process of protein anabolism, whereas ACTH is a protein catabolic agent. The analysis of body composition of the treated rats should furnish additional evidence to substantiate this conclusion.

The earlier work of Lee and Shaffer (1934) and others has already shown that growth extract of the anterior pituitary induces marked changes in the composition of the gain in body weight. In general, their results indicate that pituitary-treated rats had more protein and less fat than the controls. Our experiments employing pure growth hormone confirm these findings.

The analysis of normal giant rats cited earlier indicates that an increase of water and protein and a decrease of fat occurred in both carcass and skin of rats as the result of growth-hormone treatment (Li, Simpson and Evans, 1948). Similar experiments in hypophysectomized rats give the same results. It was found that the ACTH effect is opposite to the results secured by growth hormone injections, that is, ACTH causes the increment of fat content and a decrease of water and protein in the tissues of treated rats. The most striking change is in the fat component. This appears in agreement with the recent findings that adrenalectomized rats have less fat in their bodies (Schiffer and Wertheimer, 1947).

The changes in chemical compositions as the result of the treatment of growth or adrenocorticotropic hormone not only occurred in the carcass of the animal, but was also found in other organs of the body. The analytical data of the thymus of growth-hormone treated rats show that growth hormone induces the thymus gland to contain more water and protein and less fat. It should be noted that the average thymus weight per 100 gm body weight of the treated animals is also found to be much higher than that of the controls. In fact, it is already known that the growth-promoting activity of a pituitary extract is always coincident with the enlargement of the thymus gland of either normal or hypophysectomized animals, and that adrenocorticotropic hormone produces a decrease in the size of the thymus gland and other lymphoid tissues. (Summarized by Li and Evans, 1947).

The influence of these two hormones on the composition of liver is similar to that observed in carcass. Again in these experiments, the striking difference between the control and treated groups is the marked changes of fat content: Growth hormone reduces and ACTH increases the amount of fat in the liver. There is also an increase of water content of the growth-hormone treated liver and a decrease of water content in the livers of ACTH treated animals.

Although there appears to be no effect on the percentage content of protein in the liver treated with growth hormone, the total amount of hepatic protein in the animal is actually elevated. This is due to the increase in the size of the liver. It may be mentioned that the fact that growth hormone causes a disproportionate enlargement of liver size in normal rats was not observed in hypophysectomized animals.

The experiments of Koger, Hurst and Turner (1942), cited earlier, have shown that virgin female mice injected subcutaneously with small dosages of crystalline thyroxin stored more protein and gained more body weight per unit of food intake than did the controls during the first 27 days of treatment, whereas the controls stored more fat. There is no data available on the body composition of animals treated with a combined mixture of growth and thyroid hormones. It may be expected that these animals must store more protein and less fat than the animals receiving only either one of these hormones.

V. Plasma Non-protein Components

Recent results show that growth hormone reduces the blood free amino acids level, while adrenocorticotropic hormone elevates it (Li, Geschwind and Evans, 1948). Since changes in the level of free amino acids in the plasma may be caused by factors influencing protein metabolism, these changes may be interpreted as the result of protein anabolism and catabolism, respectively, by growth and adrenocorticotropic hormones.

It is known that plasma alkaline phosphatase level decreases with the age of the animal. There is also evidence indicating that alkaline phosphatase is related to the process of growth (see reviewed by Moog, 1946). Experiments in rats treated with growth and adrenocorticotropic hormones showed that adrenocorticotropic hormone reduces the alkaline phosphatase content in the plasma of both hypophysectomized and normal rats and that the effect is neutralized by growth hormone injection (Li, Kalman and Evans, 1947).

Clinical data has shown that the serum inorganic phosphorus level is higher in both acromegalic patients and growing children. In addition, young growing animals have higher inorganic phosphorous concentration in the plasma, and hypophysectomy

causes stoppage of body growth with accompanied lowering of the phosphorus level. It was found that growth hormone is partly responsible for such changes in the plasma inorganic phosphorus level (Li, Geschwind and Evans, 1948).

VI. Other Evidence Indicating the Protein Anabolic Nature of Growth Hormone

Desoxyribonucleic acid is found exclusively in the nucleus while ribonucleic acid is present mainly in the cytoplasm. Caspersson and others (see review by Caspersson, 1947) found that rapidly growing cells in yeast and in the root-tips of plants contain more ribonucleic acid than resting cells. Their observations appear to be true also in the case of embryonic liver, kidney, and intestine (see review by Davidson, 1947). Caspersson, therefore, concluded that since high concentrations of nucleic acid in the cytoplasm are associated with cells of growing tissues, ribonucleic acid may be in some way concerned with protein synthesis.

Since growth hormone has been shown to produce true growth effects in the tissues of rats, it would be likely that the hormone may also cause an increase in the nucleic acid content of the animal tissues. Our studies indicate that growth hormone indeed induces a small rise in the ribonucleic acid concentration in the liver, although no change in the content of desoxyribonucleic acid was observed. These results may be taken as an indication for the protein anabolic nature of growth hormone.

Although it is quite certain from our present knowledge that one of the important functions of growth hormone is to promote protein anabolism, there is a possibility that the hormone actually inhibits protein catabolism so that the gross effect appears to catalyse protein synthesis. Perhaps the most promising approach to this problem is the study of intermediate metabolism employing tracer technique. The success of this technique in the understanding of glycogen synthesis is well known.

The pioneer study of Schoenheimer and Rittenberg (1940) has demonstrated that proteins in the body are always in dynamic state, that is, amino acids in the protein molecule could be readily exchanged with free amino acids. In recent years, the rate of this exchange can easily be measured by the tracer technique. It is assumed that

the rate of this exchange (or the specific activity) in the tissue is a measure of protein synthesis. Progress of such experiments in the study of growth hormone action is just beginning. Friedberg and Greenberg (1948) have recently shown that the incorporation of sulfur from radio-methionine into the protein of skeleton muscle of mice and hypophysectomized rats, 6 hours after injections of growth hormone, increased about 70 per cent as compared to the controls; thus, the authors concluded that growth hormone exerts its effects by stimulating the incorporation of amino acids into proteins, that is, by actually accelerating protein anabolism.

VII. Conclusion

It is evident from the foregoing that the anterior lobe of hypophysis secretes two types of growth-promoting substance: one concerns the growth and development of specific endocrine glands and the other body growth as a whole. The interplay of these hormones on the growth process of an animal is clearly demonstrated and may be summarized as follows.

(1) Thyrotropic hormone or the thyroid hormone enhances the growth-promoting activity of the growth hormone. The thyroid hormone itself also promotes growth in certain animals.

(2) Adrenocorticotropic hormone is a growth-inhibiting agent in addition to its specific action on the adrenal cortex. It inhibits the growth rate of the growing animals and counteracts the growth-promoting activity of the growth hormone.

(3) Pituitary gonadotropic hormones of a male animal may produce synergistic influence on the action of growth hormone, as the male hormone is known to be a protein anabolic substance. This, however, has not been demonstrated.

I have outlined in figure I a diagram indicating the possible point of action of these hormones. It is assumed that the liver is the organ which furnishes the necessary enzymes or other catalysts for the formation of tissue proteins due to reactions between simple carbon compounds and nitrogen-containing substrates. One of the functions of growth hormone may be concerned with the break-

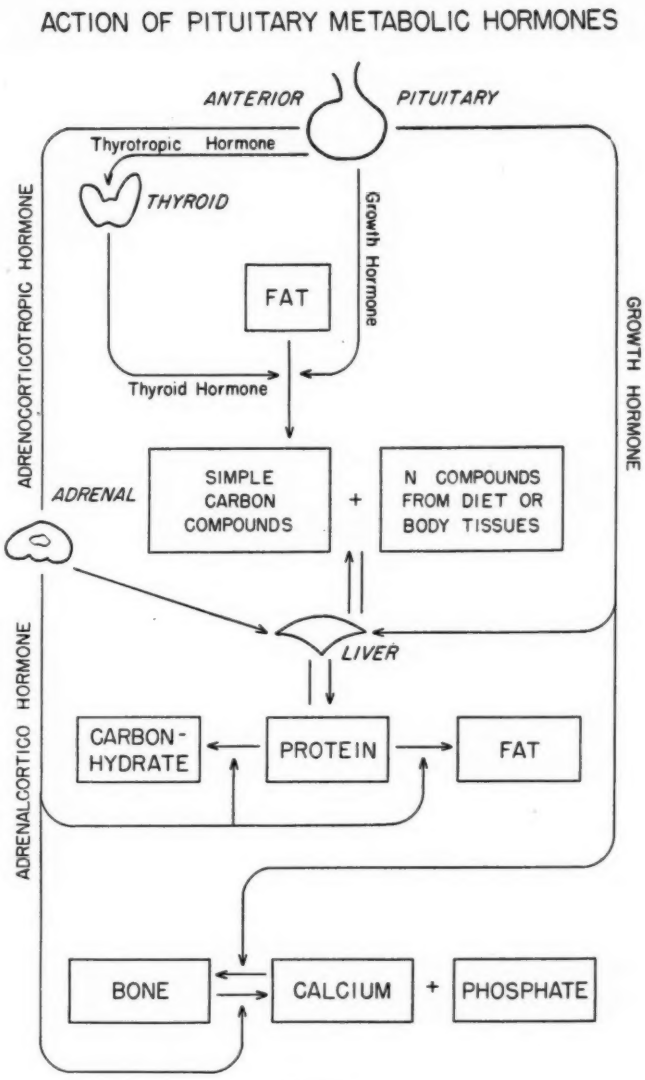


FIGURE 1.
Action of Pituitary Metabolic Hormones

down of fat into simple carbon compounds. The synergistic action of the thyroid hormone may also be assumed to catalyse fat catabolism. When the concentration of simple carbon compounds is increased, the activity of growth hormone is naturally enhanced, due to the increase in the amount of one of the substrates for protein synthesis. On the other hand, ACTH prevents fat catabolism and promotes carbohydrate formation from protein; thus, ACTH inhibits body growth and counteracts the action of growth hormone.

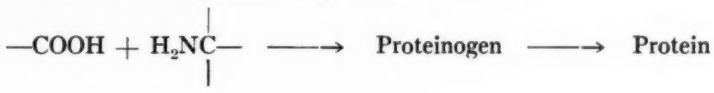
The fact that growth hormone is a protein anabolic factor seems to be established. In summary, growth hormone has been shown to:

(1) retain urinary nitrogen,
(2) lower blood amino acid,
(3) elevate protein content and decrease fat content in the carcass, thymus and liver,
(4) elevate blood alkaline phosphatase and inorganic phosphorus levels,
(5) elevate the ribonucleic acid content of the liver, and
(6) enhance amino acid uptake into protein in the skeleton muscle.

Through what mechanisms that growth hormone performs these protein anabolic process is unknown. There are many paths by which growth hormone may participate in the reaction of protein synthesis. The first step of protein synthesis is the formation of peptide bond:

It has been suggested that this reaction is coupled with an oxidative, energy-yielding reaction. It is possible that growth hormone is actually concerned in the coupled reaction. For instance, growth hormone may in some way produce, "energy-rich phosphate bonds" as the source of biologic energy for the synthesis of proteins.

Northrop (1946) has recently proposed the proteinogen hypothesis to account for the synthesis of proteins:



It was assumed that the formation of protein from proteinogen is a catalytic reaction. Is it possible that growth hormone is one of those catalysts to promote protein formation from its precursor? Since no one as yet has been able to isolate proteinogen, there is no way to ascertain this possibility.

The recent work of Cohen and McGilvery (1946, 1947) has shown that the synthesis of peptide bond is an enzymatic reaction with the supply of energy by oxidative processes. It is tempting to think that growth hormone may act as a potential enzyme or as the essential protein part of some enzymes responsible for the protein synthetic process. Green (1941) has earlier proposed similar postulation for the hormone action, as he called it, trace substance-enzyme thesis. If this thesis is correct, the antagonistic action of adrenal cortical hormones on growth hormone may then be assumed to owe their action in inhibiting growth to their competition with growth hormone in an essential metabolic reaction.

It is also probable that the mode of action of growth hormone is to produce a new enzyme *in vivo* which is especially active to catalyse the synthesis of protein. There is so far no evidence indicating that the hormone is active *in vitro*. Regardless of the exact mechanism through which the growth hormone acts, it is certain that the hormone is acting catalytically at certain steps in the synthesis of protein.

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EXTRINSIC CONTROL OF MORPHOGENESIS AS ILLUSTRATED IN THE METAMORPHOSIS OF INSECTS

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INTRODUCTION

Although morphogenesis is normally coupled to the energy yielding systems of the cell, there are occasions during the life-span of certain organisms when the gears are disengaged and the metabolic motor is left idling. These instances of spontaneous, developmental arrest have been termed "diapause". With the onset of diapause growth and differentiation cease and the animal, thereafter, persists in a genuine state of developmental standstill. With the termination of diapause the rapid tempo of cellular events returns and development continues where it had left off. Thus, whatever may be the inner mechanism for the production of diapause, it must have the net effect of dissociating the morphogenic mechanism from the mechanism of maintenance.

Among organisms diapause is not a rare phenomenon. It may intervene at virtually any stage in the life history. The dormant state that interrupts the development of the ovum prior to fertilization may be regarded as an instance of extremely precocious diapause. And, characteristically, it occurs at a precise moment — in the mammalian ovum, for example, at a stage when the chromosomes are poised in the metaphase of the second maturation division. One of the primary functions of the spermatozoan is to trigger the termination of this preembryonic diapause. From this point of view, the absence of ovular diapause is a distinguishing feature of natural parthenogenesis.

Diapause may also punctuate embryonic development, as in the case of a number of mammalian species, such as armadillos, badgers,

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martens and the roe-deer. In these instances there is evidence that its termination is evoked by progesterone (Hamlett, 1935).

Postembryonic diapause is characteristic of many species of insects and is generally terminated after exposure to low temperatures, or, in the so-called estivations, after exposure to low humidities.

It is indeed among insects that the phenomenon attains its broadest expression. Here the occurrence of diapause at some stage in the life history is, in the vast majority of species, the rule rather than the exception. It is the primary factor that determines the number of generations that a species may undergo during a growing season.

As may be observed in Table 1, diapause can intervene at any stage in development, but, for a given species, the exact moment

TABLE 1
EXAMPLES OF DIAPAUSE AT VARIOUS STAGES IN THE LIFE-HISTORY

Diapausing Stage	*Insect*
Embryo	*Bombyx, mori,* many grasshoppers
Larva just prior to emergence from egg	Tent caterpillars
Larva just after emergence from egg	Fritillary butterflies
Partially grown larva	Japanese beetle
Fully grown larva	Codling moth
Prepupa	Saw-flies
Pupa	Many species of butterflies and moths
Adult	Certain weevils

is characteristic and predictable. Moreover in certain species such as the commercial silk-worm, *Bombyx mori,* there are true-breeding races in which diapause occurs, respectively, in each generation, in each second generation, or in each third generation.

To the student of life histories the phenomenon of diapause thus presents as many problems as there are species of insects. But to the physiologist the central problem is to discern the mechanism of dormancy at the cellular level — to discover the nature of the mechanism that converts exuberant growth into developmental standstill.

This is not a modest objective since its adequate solution would require more information than we possess or are likely to obtain in the near future. Nevertheless, during recent years some progress has been made as a result of intensive study of the diapause of

several species of insects. In the next few minutes I propose to consider what we have learned concerning the mechanism of diapause in the giant silkworm, *Platysamia cecropia,* and its near relatives among the Lepidoptera.

Diapause in the Cecropia Silkworm

The eggs of the Cecropia silkworm are oviposited in early summer and about two weeks later hatch into a corresponding number of minute caterpillars. These grow rapidly, undergo a sequence of four larval moults, and finally become mature caterpillars. A few days thereafter each caterpillar spins a cocoon of silk. Within the cocoon it then sheds its cuticle and becomes a pupa.

Inside the pupa the adult moth is destined to develop. But before this occurs there intervenes a prolonged period of pupal diapause. For although the pupa may be formed in early summer, when the temperature is eminently suitable for growth, it undergoes no development for many months thereafter. If the pupa is stored at room temperature, diapause persists for five months or longer. The easiest way to terminate dormancy is to expose the pupa to low temperatures for about eight weeks. Low temperatures have physiological effects that permit the animal to initiate its development within about two weeks after return to warm temperature. For this reason exposure to cold during the months of winter guarantees that diapause will be terminated the following spring and that the adult moth will be formed within the pupa.

Over-All Control of Diapause

If we interpret this life history in physiological terms, one implication is self-evident: there must be some overall mechanism of control whereby the general bodily tissues are synchronously converted from activity to dormancy and then back to activity.

Two mechanisms seem available for this regulation. On the one hand, diapause might result from a "developmental block" due to the *presence* of an inhibitory factor. On the other hand, one might attribute the cessation of development to the *absence* of a necessary growth factor.

Fortunately, the properties of diapause in the Cecropia silkworm permit a simple experimental approach to this problem. For, by

exposing some pupae to low temperatures and maintaining others at room temperature, two types of pupae are obtained; namely, chilled pupae that are able to develop promptly at room temperature and unchilled pupae that are unable to develop promptly at room temperature. We may now consider the physiological differences between these two types of animals.[2]

Parabiotic Experiments

After removing a disc of integument from each animal, pairs of pupae were joined in parabiosis and the line of junction sealed with melted paraffin. Since the blood of insects is not contained within blood-vessels but is free in the body cavity, a direct communication was thereby established between the pupae in each pair so that their blood might be continuously shared. When diapausing pupae were paired in this way with previously chilled pupae, a test of the two principal theories of diapause was provided. For, if the diapausing pupa was arrested in development due to the presence of some inhibitory factor in its blood, then this factor should pass to the previously chilled pupa to block its development also. In contrast, if the diapausing animal lacked some necessary growth factor and was thereby prevented from developing, one might expect the previously chilled pupa to provide this factor so that both animals would develop.

The experimental results supported the second interpretation, for the animals in each pair underwent prompt and simultaneous development. Thus these initial experiments provided substantial evidence that diapause was consequent to the lack of a growth factor which could be produced after a period of chilling.

Similar parabiosis between diapausing Cecropia pupae and previously chilled Polyphemus pupae yielded identical results, although these species belong to different genera. The growth factor necessary for adult development must, at least to this degree, lack species-and-genus-specificity.

One noteworthy feature of all such parabiotic preparations is that the two pupae in each pair invariably grow together at the site of grafting. This occurs even when a plastic tube as long as three centimeters is interposed.

[2]Some of these experiments have been reported previously in greater detail (Williams, 1946, 1947, 1948).

The Role of the Brain

Since previously chilled pupae are thus able to produce a factor that terminates diapause, a search was made to discover the source of such a factor within the previously chilled pupa. For this purpose various tissues and organs were removed from previously chilled pupae and implanted singly into diapausing individuals. These experiments revealed that only one organ in the chilled pupa has the power to evoke the development of diapausing pupae and that this organ is the brain itself. For when the brain of a previously chilled pupa is implanted into the head, thorax, or abdomen of a diapausing pupa, diapause is terminated and adult development ensues. This occurs even when the chilled brain is obtained from closely related species and genera.

In contrast to the effectiveness of the brains of previously chilled pupae, brains removed from diapausing pupae are either ineffective or able to terminate dormancy only after a long latent period of at least five months.

Time does not suffice to describe a number of other experiments that pertain to this action of the brain, but we may summarize by saying that the brain-factor is indispensable in these species for the termination of diapause. If the brain is removed from a diapausing pupa, the animal may continue to live for two years thereafter, but the diapause is permanent.

The Effect of Temperature

The evidence, considered up to this point, supports the conclusion that diapause results from a failure of the brain to produce a growth factor that is necessary for further development. The reason that exposure to low temperature is effective in terminating diapause is that low temperature renders the brain able to produce adequate amounts of this growth factor. The other tissues of the diapausing pupa do not require chilling, for they are promptly activated by implanting a brain which, alone, has been chilled.

Consequently, the activation of the pupal brain during exposure to low temperature must consist of some physical or chemical alteration in its substance. The primary effect is to render the brain competent to release its factor during subsequent exposure to developmental temperatures. Actual release of the factor seems to occur

slowly during the period of chilling, but rapidly if the pupa is then placed at high temperatures. A critical period occurs about two weeks after return to 25° C. and is signalled by the first indications of adult development. Simultaneously, the brain becomes inactive and metamorphosis can proceed without its further participation.

Neurosecretory Nature of Brain Function

We may now consider what has been learned about the site of origin of the growth factor within the brain. For this purpose brains were removed from previously chilled pupae and subdivided into fragments. The activity of each fragment was then tested by implanting it into a brainless, diapausing pupa.

The pupal brain (Figure 1) may be conveniently divided into a

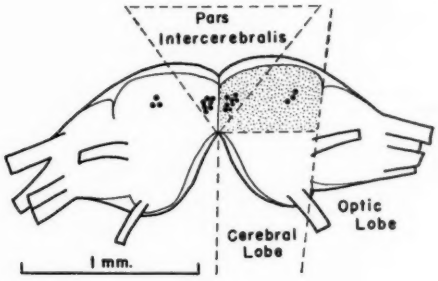


FIGURE 1.
Diagram of the living pupal brain of the Cecropia silkworm. The region that produces the brain-factor lies in each hemisphere within the inner mass of the cerebral lobes (shaded zone in the diagram). This region contains the medial and lateral groups of neurosecretory cells.

pair of cerebral lobes and a pair of optic lobes. It may further be divided into an outer and an inner mass. In the living brain one can see at the periphery of the inner mass, a number of large ganglion cells which, in histological section, show pronounced signs of secretory activity. In each hemisphere they are arranged in two groups: a medial group of eight cells and a lateral group of about three cells.

When the various parts of the brain are tested for activity, the only active fragment is the inner mass of the cerebral lobes containing these neurosecretory cells. Both medial and lateral groups seem to

be indispensable, for the medial groups of the pars intercerebralis are inactive by themselves, even when such fragments are implanted in large numbers. The lateral group, by themselves, are similarly inactive. Both medial and lateral groups seem to be necessary. It is also worth noting that the two groups apparently require organic connection with one another, for activity seems to be lost when the medial and lateral groups are cut apart. This suggests that the two groups of neurosecretory cells may collaborate to produce a single developmental factor.

The Nature of the Brain-Factor

In view of the extraordinary action of the brain-factor, its nature is consequently of great interest. Progress in this direction has been disappointing. We might assume the factor to be a blood-borne hormone, yet blood removed from pupae at various stages in development has invariably proved inactive. If the brain-factor is a blood-borne hormone, then it must either be extremely unstable or present at any one moment in low titer.

Of interest in this connection is the fact that only living brains have been found active. Thus brains killed by freezing or by exposure to high temperatures are invariably inactive, even when implanted in large numbers. It may also be noted that an implanted brain connects up with the tissues of the host before exerting its effect. These connections present the possibility that the brain-factor may travel in the tissues rather than in the blood.

The several lines of evidence, up to this point, are consistent in demonstrating for the Cecropia silkworm an over-all control of pupal diapause by mechanisms that are of endocrinological interest. Diapause seems to consist of an interruption in the normal processes of development by virtue of a failure of the brain to supply a non-species-specific factor necessary for adult formation. Diapause is terminated when adequate amounts of this factor are provided.

The Role of the Prothoracic Glands

It was a rational assumption that the brain-factor acted on the diapausing tissues to convert them to activiy. We may now briefly consider certain experiments that demonstrate the inadequacy of this assumption.

From a considerable number of pupae the terminal six abdominal segments were excised. Both anterior and posterior fragments of the subdivided pupa were then sealed onto plastic cover-slips by means of melted paraffin. Many of these fragments continued to live for as long as eight months thereafter and at any time during this period it was easy to accomplish implantations or transfusions by a way of a paraffin-plugged hole, centrally placed in each plastic slip.

Now if the anterior fragment possessed or was provided with a previously chilled brain, it developed into the corresponding anterior end of an adult moth. In contrast, isolated abdomens could not be induced to undergo development by brain implantation. As many as six previously chilled brains have been implanted into a single isolated abdomen without inducing development.

Consequently, it is evident that anterior and posterior fragments respond differently to brain implantation. This difference could be explained if the anterior end possessed a second center that, in addition to the brain, was necessary for adult development.

To test this possibility the level of section was varied so as to include in the posterior fragment more and more of the anterior region of the pupa. A critical level of section was discovered which included the pupal mesothorax. Such a posterior fragment can be induced to develop by implanting a chilled brain. From these observations we may conclude that a second center does exist in the anterior region of the pupa and, most probably, in the thorax.

A search was therefore made to locate a thoracic organ that might collaborate with the brain to induce adult development. For this purpose a chilled brain, plus various thoracic organs, were implanted into isolated abdomens. This search was vastly aided by the publication of Fukuda's paper in 1941. As a result of experiments on *Bombyx mori,* this Japanese investigator reported an endocrinological function for an organ which had hitherto been overlooked, the "prothoracic glands".

It soon became evident that these glands were, indeed, the long-sought, thoracic differentiation center. For when a chilled brain, plus prothoracic glands, were implanted into an isolated pupal abdomen, the abdomen was induced to undergo complete adult development.

It is worth emphasizing that development of these isolated abdomens cannot be brought to pass by the prothoracic glands alone. A previously chilled brain must also be implanted.

One can reduce such isolated abdomens to the barest minimum consistent with survival, and a brain plus prothoracic glands may still produce complete adult development. Thus, one can remove the entire digestive tract, the Malpighian tubules, the gonads, and the entire central nervous system and the isolated abdomen can still metamorphose when a brain plus prothoracic glands are implanted.

Interaction Between Brain and Prothoracic Glands

It is evident from these experiments that the termination of pupal diapause requires a minimum of two factors, one from the brain and the other from the prothoracic glands. The easiest way to render these organs functional is to expose the diapausing pupa to low temperatures and then return it to room temperature. As previously mentioned, the effectiveness of chilling in terminating dormancy can be explained in terms of an action of low temperature on the brain, whereby the brain is made competent to release its developmental factor. Manifestly, the prothoracic glands do not require similar exposure to cold, for they are promptly activated when a chilled brain is implanted into a diapausing pupa.

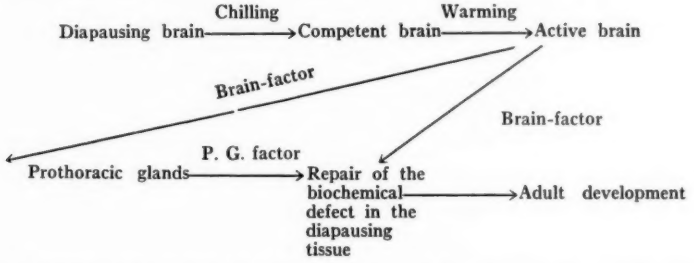
From these observations we may conclude that the brain exerts a controlling action on the prothoracic glands. Subsequent experiments have confirmed this hypothesis consistently. Thus, to induce the development of isolated pupal abdomens, it is necessary that the implanted brain be obtained from a previously chilled pupa. This is not the case in regard to the implants of prothoracic glands, for these organs are equally effective when obtained from diapausing pupae. The functional failure of the prothoracic glands at the outset of diapause seems therefore to result from a primary failure of the brain in releasing its factor.

The mechanism that terminates diapause must ultimately supply the dormant tissues with something that they require for cellular growth and differentiation. In respect to this process the experimental results may be interpreted from two points of view. On the one hand, the brain-factor may be conceived as having sole action on the prothoracic glands and the prothoracic gland factor, in turn, as having specific effect on the tissues of the body in general. On the other hand, the tissues may require interaction with both factors, the first factor serving to condition the tissues for reaction with the second.

The evidence at hand does not suffice to exclude either of these interpretations. One aspect seems sufficiently clear, however: the factor having ultimate action on the dormant tissues to convert them to activity is the factor from the prothoracic gland. Thus, we have noted that adult formation becomes independent of the brain as soon as the earliest signs of adult development are evident. However, for about five days thereafter, the further progress of morphogenesis continues to depend on the function of the prothoracic glands. Until the insect reaches a stage characterized by the initiation of eye pigmentation, its cells require a supply of the factor from the prothoracic gland. Only at this relatively late stage in morphogenesis does their differentiation become autonomous and independent of over-all control.

Consequently, the primary control that the brain exercises over diapause is accomplished, at least in part, by an indirect mechanism; namely by the action of the brain-factor in triggering the functional activity of the prothoracic glands.

The release of the brain-factor sets in motion a sequence of reactions that may be summarized as follows:



Obviously, the point of primary interest is the reaction that occurs in the tissues. As a working hypothesis we may assume the existence of some kind of biochemical defect in the diapausing cell that prevents its metabolism from contributing to morphogenesis. From this point of view, the brain and prothoracic glands should preside over some synthetic reaction whereby the cell repairs this defect.

The metabolism of pupae during diapause and during development is therefore of interest. As might be anticipated, the rate of oxygen consumption of diapausing pupae is low. A relatively low metabolism also characterizes chilled pupae during the first ten days after return to room temperature (Figure 2). A striking peculiarity of the metabo-

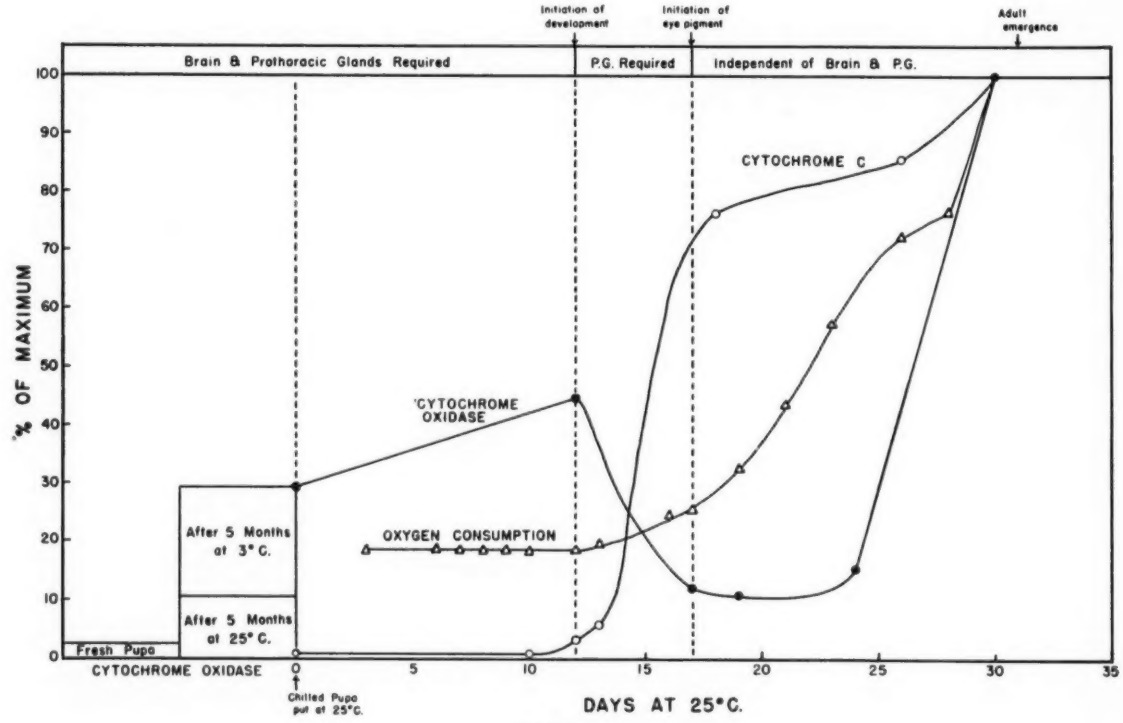


FIGURE 2.

The oxygen consumption and titers of cytochrome oxidase and cytochrome *C* during diapause and development in the Cecropia pupa.

lism of these undeveloped pupae is its insensitivity to cyanide. When the brain and prothoracic glands function to terminate diapause, the metabolism begins to increase (Figure 2). A progressively larger fraction of the metabolism then becomes cyanide sensitive.

These observations are of interest since they indicate that the metabolism during diapause may differ qualitatively from the metabolism during development. Development seems to involve a progressively larger utilization of the cytochrome system.

Two hypotheses may be advanced to explain these alterations. During diapause the metabolism may be conceived as diverted to pathways that leave the cytochrome system intact, but uncoupled. With the termination of diapause the system could once more be utilized. Alternatively, the cytochrome system may be viewed as broken down at the outset of diapause and resynthesized at the outset of development.

To test these two possibilities, spectroscopic studies were performed on larvae, pupae, and adults of the Cecropia silkworm. These studies revealed that the cytochromes underwent dissolution just prior to pupation and were virtually absent in the diapausing pupa.

Subsequently, the measurements[3] have been repeated on a quantitative basis by spectrophotometric assay of the cytochrome C content of individual animals. A striking correlation was found between the concentration of cytochrome C and the progress of development (Figure 2). During the period when development is under the control of the prothoracic glands, the concentration of cytochrome C increases from less than one gamma to more than 50 gamma per gram live weight.

To these results may now be added a brief summary[3] of comparable changes that occur in cytochrome oxidase. During the period of the brain's secretory activity, the titer of cytochrome oxidase increases from approximately 40 to nearly 700 units (Figure 2). That this increase is dependent on the function of the brain is indicated by two lines of evidence: (1) the increase does not occur if the brain is removed from diapausing pupae, and (2) removal of the brain at any stage prior to the initiation of development is followed by a decay of cytochrome oxidase activity to the old diapausing level.

[3]These chemical studies were performed in collaboration with Mr. R. C. Sanborn and Mr. M. S. Grossman and will be described elsewhere in greater detail.

When a chilled pupa is placed at room temperature, a rapid increase occurs in the titer of cytochrome oxidase. The brain, as we have seen, then triggers the prothoracic glands and development is promptly initiated. Simultaneously the brain becomes inactive. It is, therefore, of interest that this inactivation of the brain is followed by an abrupt decrease in the titer of cytochrome oxidase. This decrease continues until the animal reaches the advanced stage beyond which development can continue without further "hormonal" control. At this stage the cells of the general bodily tissues seem to become self-sufficient in their synthetic ability, for, in the complete absence of brain-factor, the titer of cytochrome oxidase now undergoes rapid increase to the level characteristic of the adult moth.

From these tedious chemical analyses two primary correlations emerge: namely, that between the synthesis of cytochrome oxidase and the function of the brain, and that between the synthesis of cytochrome C and the function of the prothoracic glands. As a result of the combined functions of both the brain and the prothoracic glands, the tissues of the diapausing pupa, for the first time, come into possession of a complete cytochrome system.

It is an attractive theory to regard the absence of the cytochrome system as the basic biochemical defect in the diapause of these species. Yet such a conclusion is not justified on the basis of the evidence we have just considered. Obviously, the alterations in the cytochrome system might be a result rather than a cause of development. Time does not suffice to describe a variety of experiments that have been performed to test these possibilities. Suffice it to say that studies involving prolonged exposure to high tensions of carbon monoxide and of HCN have yielded inconclusive results. In the presence of these agents previously chilled pupae have been observed to initiate development and then to die within a few days thereafter. Although development is delayed and never proceeds beyond its initial stage, it has definitely been initiated under conditions where we would expect a large fraction of iron-catalyzed oxidations to be inhibited.

It would therefore be premature to identify the cytochrome system as the missing link in the metabolism of diapause—the changes which it undergoes may be secondary to more basic alterations in other enzyme systems. In any event, we have a rough outline of the type of mechanism we may anticipate—an outline in which the cell is not a free agent in its program of morphogenesis.

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EFFECTS OF ULTRAVIOLET RADIATION ON CELLS*

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I. INTRODUCTION

Research on the biological effects of ultraviolet radiation has been concerned largely with such practical problems as the control of airborne microorganisms, the use of ultraviolet in radiation therapy, and the evaluation of radiation hazards. These practical problems will not concern us; instead, our purpose will be to consider the interaction of ultraviolet radiation with cells from the special point of view of the information gained therefrom about fundamental biological phenomena, including cell division, genetic processes, growth, differentiation, and cell metabolism.

1. *Dual Nature of the Interaction of Radiation with Cells*

The information gained about biological phenomena by the use of ultraviolet radiation arises both from effects of radiation on cells and from effects of cells on radiation. Thus, when radiation inhibits the division of cells, absorption of radiant energy is involved, and the result of such absorption is to change the incident radiation in intensity or spectral energy distribution or both. On the other hand, when radiation is used to study cytochemical problems by means of the absorption spectra of portions of cells, the absorbed radiation always gives rise to changes of one kind or another in the cells.

The dual nature of radiobiological processes is important for two reasons: First, the changes in radiation that accompany the modification of biological processes by radiant energy often provide information of use in understanding these processes. For example, one may

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determine the relative effectiveness of different spectral ranges of the ultraviolet in inducing mutations; the so-called *action spectrum* obtained in this way may then be used to deduce the nature of the chemical substances involved in the process. Second, the modifications produced in biological systems when radiation is used to gain information about them inevitably result in some degree of indeterminacy in the observations. Thus, one cannot use the ultraviolet microscope to determine the absorption spectrum of a portion of a cell without modifying the substances responsible for the absorption. A clear realization of this limitation is necessary if unjustified interpretations are to be avoided.*

2. *Plan of Presentation*

The fundamental physical and photochemical basis of the interaction of ultraviolet radiation with cells will be stressed throughout this discussion. This approach will permit a more quantitative and critical review of the field than would otherwise be possible, but will also necessitate less emphasis on the descriptive biological aspects of the subject. The latter are adequately covered in numerous reviews listed subsequently. It seems most profitable to emphasize here important phases of the subject that have received little attention elsewhere.

We may group the investigations to be described into three general categories according to whether their primary purpose is: (1) to block or accelerate specific biochemical or physiological processes in cells, (2) to modify cells more generally and non-specifically, or (3) to obtain cytochemical data. In the first and second instances, emphasis is upon the effects of radiation on cells; in the third, it is upon the effects of cells on radiation.

*All observations involve processes that modify the systems observed. In physics, this concept is known broadly as the ***principle of indeterminancy*** or in application to observations at the atomic level as ***Heisenberg's uncertainty principle***. One does not therefore attempt to obtain observational methods that will have *no* effect on the observed system. Instead, one endeavors to evaluate the indeterminancy or to choose methods in which the indeterminancy is small in comparison with the desired accuracy of the observations.

II. Physical and Photochemical Basis of the Interaction of Ultraviolet with Cells

1. Relation of Spectral Regions to the Interaction of Radiation and Matter

The biological effects of electromagnetic radiation are usually attributed, directly or indirectly, to one of two causes: (1) conversion of radiant energy to heat with rise in temperature of the irradiated specimen, or (2) primary or secondary chemical effects arising from the interaction of radiation with matter (Fig. 1).

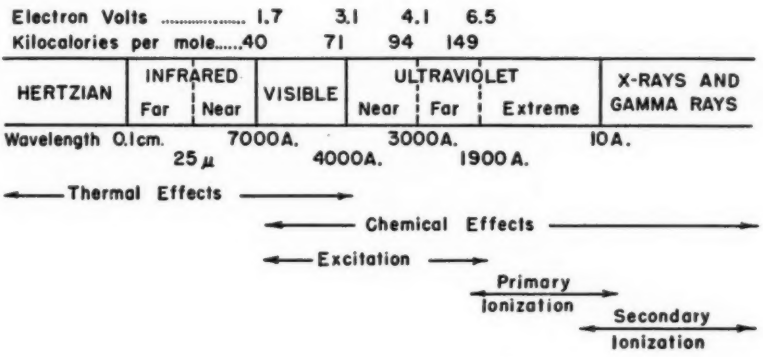

FIG. 1.

Chart of the Electromagnetic Spectrum (Not to Scale) Showing the Principal Atomic and Molecular Effects of Radiation to Which Biological Effects Are Attributable

Thermal effects are particularly important in explaining the biological action of radiation of comparatively long wavelength (including Hertzian, microwave, and infrared radiation and, to a lesser extent, visible radiation), and become of progressively less consequence toward shorter wavelengths. In the x-ray and gamma-ray regions, thermal effects are negligible in comparison with other phenomena. Chemical effects occur to some extent in the visible region (a common example in biological systems is photosynthesis) but are more generally characteristic of shorter wavelengths, beginning particularly with the far ultraviolet.

Among the chemical effects observed are photodecomposition,

photosynthesis, polymerization, depolymerization, and molecular rearrangements. These often involved complex sequences of events. The simpler primary processes, each of which is associated with a particular range of the spectrum, are: (1) excitation of molecules to energy levels corresponding to unstable vibrational states, (2) ionization by ejection of valence electrons, and (3) ejection, from the inner shells of atoms, of high-speed electrons that lose their energy by the production of ion pairs along their trajectories, the ions so formed subsequently participating in chemical reactions. Excitation phenomena are characteristic of the visible and near and far ultraviolet regions. Ionization by ejection of valence electrons occurs particularly in the vacuum ultraviolet. Ejection of high-speed electrons from the inner shells of atoms, with subsequent production of ion pairs, is characteristic of x-rays, gamma rays, and high-energy particles, such as alpha and beta rays, protons, and neutrons. Production of ions is so usual as a primary and secondary effect of these last forms of radiant energy that they are frequently called *ionizing radiation.*

Our concern is with ultraviolet radiation, hence with photobiological phenomena attributable to photochemical effects that arise from the excitation of molecules. As shown in Fig. 1, ultraviolet radiation ranges from about 4000 to 10 A., starting at the extreme blue end of of the visible spectrum and overlapping the x-ray region at its shortest wavelengths. Physicists subdivide the ultraviolet as follows:* (1) the *near ultraviolet,* near the visible, from 4000 A. to 3000 A., the approximate short-wave limit of solar radiation at the earth's surface; (2) the *far ultraviolet,* from 3000 A. to 1900 A., the approximate short-wave limit of transmission of quartz and of atmospheric oxygen; and (3) the *extreme* or *vacuum ultraviolet,* from 1900 A. to 10 A., the approximate limit thus far reached in the excitation of ultraviolet radiation. In the extreme ultraviolet, one must ordinarily work with evacuated equipment in order to eliminate absorption of radiation by oxygen and other gases, hence the name "vacuum ultraviolet". The range of the vacuum ultraviolet from about 1900 A. to 1100 A., the approximate short-wave limit of transmission of fluorite, is often called the *Schumann region,* after the investigator who first explored it.

*Biologists and medical workers often divide the ultraviolet in accordance with a terminology adopted at the second International Light Congress in Copenhagen (see *British Journal of Physical Medicine, 7,* 124, 1932) in which the range from 4000 to 2000 A. is subdivided into three regions: the *long ultraviolet,* from 4000 to 3150 A.; the *medium ultraviolet,* from 3150 to 2800 A.; and the *short ultraviolet,* below 2800 A. These regions are characterized by substantially different biological effects.

2. *Fundamental Laws Relating the Effects of Radiation to Wavelength*

The wavelength dependence of photobiological effects can be explained by two fundamental laws: (1) *Planck's quantum law* of interaction of radiation with matter and (2) the *Grothus-Draper law.*

a. Planck's Quantum Law—For our purpose, Planck's quantum law may be stated as follows: When radiant energy is emitted or absorbed by an atom or molecule, the corresponding quantity of energy change (or *quantum*), ΔW, is directly proportional to the frequency, ν, of the radiation. Expressing this symbolically:*

$$\Delta W = h\nu \qquad (1)$$

Equation (1) is fundamental in relation to photochemical and photobiological processes. It indicates that if a change in energy $\Delta W'$ is required to dissociate a molecule, the dissociation can be induced as a direct, one-step, photochemical process only by radiation of frequency equal to or greater than ν', where $\nu' = \Delta W'/h$.

Planck's law accounts for the inappreciable photochemical and photobiological effects of Hertzian, microwave, and infrared radiation and the marked effects of ultraviolet radiation, as illustrated by Tables I and II. Table I shows the magnitudes of the photochemical energy equivalents of various wavelengths as computed from equation (1). The energies are shown in kilocalories per mole for convenience in comparing chemical bond strengths and in electron volts for comparison with ionization potentials. Table II shows thermochemical bond energies for some bond groups typical of biochemical substances. Photochemical dissociation energies are usually higher than the bond energies in Table II. Dissociation often takes place under conditions in which one or both of the end products is in an excited energy state, and the absorbed quantum must then supply the energy of excitation as well as that of dissociation. Furthermore, the energies required to dissociate bonds encountered in biochemical substances are frequently increased as a result of chemical resonance (Pauling, 1945).

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*The energy change, ΔW, is expressed in ergs and the frequency ν in cycles per second. Alternatively, one may write $\Delta W = hc/\lambda$, where c is the velocity of light in cm. per sec. (3×10^{10}, approximately) and λ is the wavelength in cms., or $\Delta W = hc\sigma$, where σ is the wavenumber, that is to say, the number of waves in a 1-cm. path in vacuum ($\sigma = 1/\lambda$, where λ is in cms.). The constant of proportionality, h, known as Planck's quantum constant, is equal to 6.624×10^{-27} erg secs.

TABLE I
PHOTOCHEMICAL ENERGY EQUIVALENTS FOR VARIOUS WAVELENGTHS OF ULTRAVIOLET AND VISIBLE RADIATION

Wavelength Angstroms	Wavenumber $cm.^{-1}$	Kilocalories per mole	Electron volts
	a. Far Ultraviolet		
2000	50,000	143	6.20
2200	45,455	130	5.63
2400	41,667	119	5.16
2600	38,462	110	4.77
2800	35,714	106	4.59
3000	33,333	95.3	4.13
	b. Near Ultraviolet		
3200	31,250	89.4	3.87
3400	29,412	84.0	3.64
3600	27,718	79.2	3.47
3800	26,316	75.2	3.26
	c. Visible		
4000	25,000	71.4	3.10
5000	20,000	57.2	2.48
6000	16,667	47.6	2.06
7000	14,286	41.8	1.81

Conversion Factors

$1\ cm^{-1} = 1.99 \times 10^{-16}$ ergs per molecule
$= 2.86 \times 10^{-3}$ kilocal. per mole
$= 1.24 \times 10^{-4}$ electron volts

(For more precise conversion factors and a discussion of units and conversion factors useful in photochemistry, see Herzberg, 1944)

TABLE II
THERMOCHEMICAL BOND ENERGIES OF SOME BONDS IN BIOCHEMICAL COMPOUNDS

Bond	Bond Energy kcal./mole
C—C	58.6
C=C	100
C—H	87.3
C—N	48.6
C=N	94
C—O	70.0
O—H	110
S—H	87.5

(Data from Pauling, 1945.)

Instances in which absorbed quanta result in *ionization* are of special interest in relation to vacuum-ultraviolet and shorter wavelength radiation. In such instances, the absorbed quanta must supply energy w_o sufficient to remove an electron from the influence of an atom or molecule, plus the kinetic energy imparted to the electron, that is to

say, $\frac{1}{2}mv^2$, where v is the velocity with which the electron leaves the atom or molecule. The situation may be expressed symbolically by Einstein's photoelectric equation:

$$h\nu = w_o + \frac{1}{2}mv^2 \qquad (2)$$

In this equation, w_o is usually expressed in *electron volts*, and is called the *ionization potential*. Table III shows the ionization poten-

TABLE III
IONIZATION POTENTIALS OF SOME ELEMENTS OF BIOCHEMICAL IMPORTANCE

Element	First Ionization Potential Electron volts
Hydrogen	13.6
Carbon	11.3
Nitrogen	14.5
Oxygen	13.6
Sodium	5.1
Sulphur	10.3
Phosphorus	10.9
Potassium	4.3

(Data from Richtmeyer and Kennard, 1942; and Handbook of Chemistry and Physics, 30th Ed.)

tials of the outermost (valence) electrons of elements of principal biological interests. The data indicate that vacuum-ultraviolet quanta are required to ionize most biologically important atoms. Photobiological effects in the vacuum ultraviolet, which have received but scant study (see Lyman, 1928, Heyroth, 1941), should therefore be of especial interest in relation to the effects of x-rays, gamma rays, and high-energy particles.

b. The Grothus-Draper Law.—The Grothus-Draper law states that only absorbed radiation is effective in bringing about photochemical change. It is essentially a special case of the law of conservation of energy, and indicates the importance of considering absorption spectra in relation to photochemical effects. Thus, even if the energy quanta are sufficiently large to initiate a given photochemical reaction, the efficiency of the reaction may be low in terms of total incident energy requirements if absorption is low at the wavelength under consideration. Photochemical changes in desoxyribose nucleic acid take place at wavelengths shorter than about 2950 A. (Heyroth and Loofbourow, 1933), but in dilute solutions and short path lengths much less total incident energy is required to produce such effects at

2600 A. than at 2300 A. because pronounced absorption maxima and minima occur respectively at these two wavelengths.

Some workers appear to assume from the Grothus-Draper law that all absorbed radiation *necessarily* produces photochemical and photobiological effects. This is obviously incorrect, since the absorbed quanta must be of sufficient magnitude if photochemical effects are to take place. Furthermore, absorbed energy may be dissipated in various ways other than photochemical change, as for example in increased thermal motion, inelastic collisions (collisions of the second kind), and fluorescence. Colored dyestuffs are everyday examples of substances having marked absorption without marked photochemical lability. These substances owe their characteristic color to absorption of visible radiation, but are usually chosen with special regard to long stability upon exposure to light.

3. *General Considerations Regarding Photochemical Processes in Biochemical Substances*

As might be deduced from the quantum values and bond energies in Tables I and II, the majority of biochemical substances that exhibit appreciable absorption in the far ultraviolet (3000-1900 A.) undergo photochemical change in this region. At wavelengths longer than roughly 2300 A., marked absorption is exhibited primarily by organic compounds containing unsaturated groupings, and in particular by compounds containing conjugated double bonds. Among such compounds are the aromatic amino acids, tyrosine, tryptophane, and phenylalanine, the purines and pyrimidines, unsaturated fatty acids, unsaturated steroids, carotinoids and other plant pigments, and numerous coenzymes, vitamins, and other biologically important substances. Saturated compounds, such as simple sugars, exhibit very little absorption at wavelengths longer than about 2300 or 2400 A., and are comparatively stable throughout most of the near and far ultraviolet, high energies being required to produce such photochemical changes as are observed. Saturated organic compounds absorb markedly in the Schumann region, and would be expected to be quite labile in this range of high-energy quanta.

The available data regarding photochemical effects on biochemical substances is largely qualitative, for two reasons. First, the quantitative evaluation of photochemical effects as a function of wavelength

requires elaborate special equipment, not available in many laboratories. Second, the determination of the effects that take place in the photodecomposition of polyatomic molecules is much more complex than in the case of simple diatomic molecules. Precise, quantitative photochemical investigations have been carried out principally with diatomic molecules in the gaseous state. The determination of their photodecomposition products involves quantitative analysis for one or two atoms or radicals of known identity, and these may sometimes be detected, and even determined quantitatively, by direct observation of spectral changes. In the case of polyatomic molecules, the situation is much more complex. A large variety of molecular rearrangements, polymerization reactions, or partial decompositions may take place, and several such reactions may occur simultaneously. The evaluation of changes in terms of end products thus becomes much more difficult. Biological criteria of photochemical change can sometimes be employed. Thus, destruction of biological activity has been used as an end point in evaluating photochemical effects on vitamins (see Uber and Verbrugge, 1940 a, b). Such end points are not necessarily clear cut, however, since changes that render the molecule inactive for one type of organism may not render it inactive for other types.

We shall not attempt to give a systematic review of the photochemistry of biochemical substances, but will summarize selected investigations of compounds of particular interest. An extensive and thorough review of the literature has been published by Heyroth (1941). The theoretical aspects of photochemistry are discussed from the chemical viewpoint by Ritchie (1941) and Noyes and Leighton (1941) and from that of the relation of photochemical processes to molecular spectroscopy by Jevons (1932) and Herzberg (1939).

4. Photochemistry of Amino Acids and Proteins

The absorption spectra of some typical amino acids and proteins are shown in Figure 2. As suggested by this figure, almost all of the absorption of proteins at wavelengths longer than about 2300 A. is attributable to the aromatic amino acids.

a. Non-Aromatic Amino Acids—Since the absorption of non-aromatic amino acids is inappreciable at wavelengths longer than about 2300 A., they would be expected to be relatively little affected in this range, and this has been found to be the case. From studies

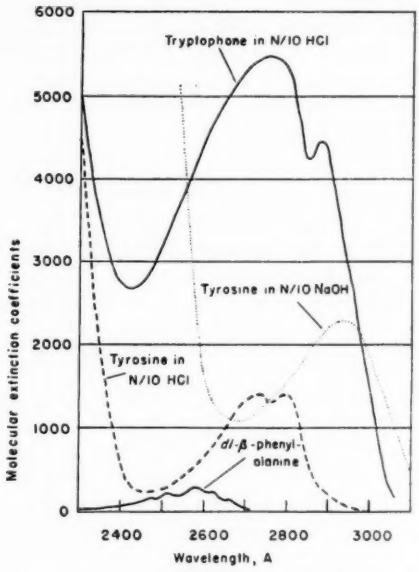

FIG. 2a.
ABSORPTION SPECTRA OF AROMATIC AMINO ACIDS (FROM COMPILATION BY LOOFBOUROW, 1940)

of dissociation spectra,* the wavelength threshold for dissociation of glycine in neutral or acid solution appears to be about 1950 A. and in alkaline solution about 2000 A. (Anslow, Foster, and Klinger, 1933.) Other investigations of the photodecomposition of non-aromatic amino acids indicate wavelength thresholds as long as 2200 A. (Henri, Weizmann, and Hirschberg, 1934 a, b). Prolonged irradiation of glycine with ultraviolet of sufficiently short wavelength leads to several decomposition and side reactions, including hydrolysis with release of ammonia (Weizmann, Hirschberg, and Bergmann, 1938). The foregoing results are typical, and indicate that the non-aromatic amino acids probably play little part in biological effects at wavelengths longer than about 2200 A.

*Especially in the case of simple gases, the spectroscopist obtains useful clues as to the wavelength ranges in which molecules are dissociated by the occurrence of continuous absorption (indicative of non-quantized energy transitions) in these regions. Such spectra are known as *dissociation spectra*. Often they are preceded at longer wavelengths by regions of diffuse bands known as *predissociation spectra*. (See Jevons, 1932; Herzberg, 1939.)

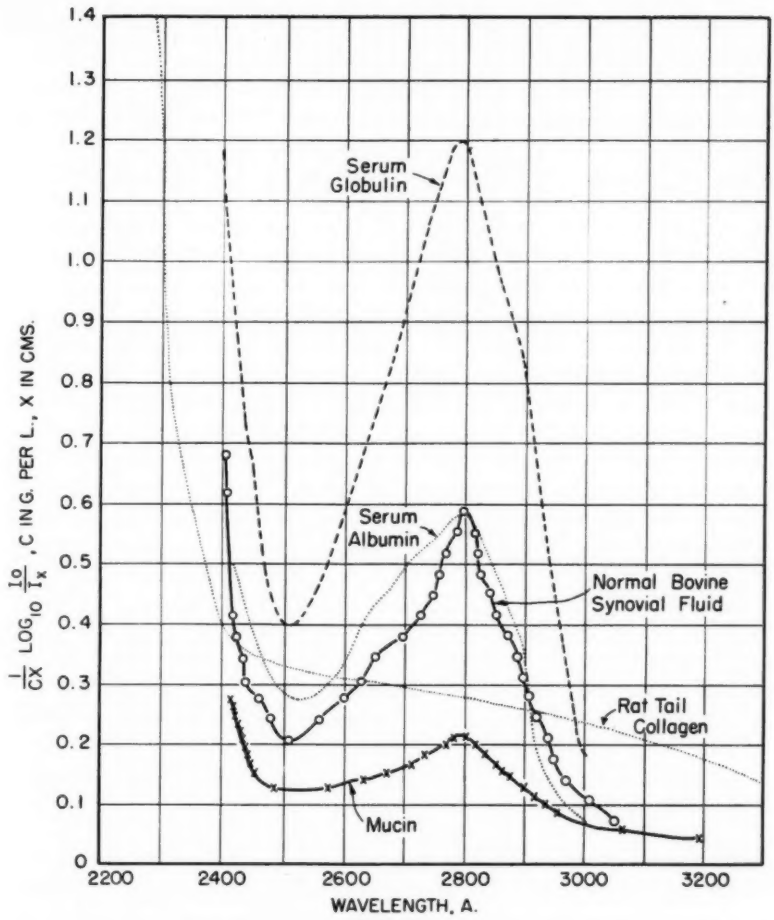

FIG. 2b.

ABSORPTION SPECTRA OF SOME PROTEINS AND PROTEIN-CONTAINING PREPARATIONS (FROM COMPILATIONS BY LOOFBOUROW, 1940, MORTON, 1942, AND UNPUBLISHED DATA OF H. LISCO AND D. G. LOOFBOUROW).

b. Aromatic Amino Acids—Aromatic amino acids exhibit characteristic absorption in the far ultraviolet and are correspondingly labile. Tyrosine and tryptophane are decomposed throughout the entire far ultraviolet region and phenylalanine through at least a considerable portion of it. Typical changes include the evolution of ammonia and an initial shift of the absorption spectrum to somewhat longer wavelengths (Magill, Steiger, and Allen, 1937; Allen, Steiger, Magill, and Franklin, 1937). Tryptophane and tyrosine take up oxygen when irradiated with far ultraviolet radiation; this is said to be the principal reason for the uptake of oxygen by irradiated plasma (Harris, 1926). Irradiation of tryptophane has been found to yield, among other products, indole-3-acetic acid (Berthelot and Amoreux, 1938). A product of the irradiation of l-tyrosine is said to be *dopa* (Arnow, 1937, 1938), a precursor of melanin.

c. Proteins—Proteins were among the first biologically important substances to be investigated photochemically (see Arnow, 1936), and the action of the far ultraviolet on cells was attributed very early to denaturation of proteins. Among the earlier studies are those of Bovie and his co-workers (Bovie, 1913; Bovie and Hughes, 1919 Bovie and Klein, 1919; Bovie and Daland, 1923). Most of their work was carried out with albumin. Typical results upon exposure to far ultraviolet at room temperature included progressive coagulation and development of a strong odor. When irradiated at zero degrees, the solutions remained clear until heated a few degrees subsequent to irradiation, whereupon coagulation occurred. Thus, there appeared to be a primary photochemical process, independent of temperature, and a secondary, thermally dependent process, resulting in coagulation.

Grossly, the results of these rather drastic radiation exposures resemble the phenomena observed in heat coagulation of proteins, although the end products do not behave analogously in all respects (Spiegel-Adolf, 1927; Spiegel-Adolf and Krumpel, 1929).

Stedman and Mendel (1926) studied the effects of irradiation on several purified proteins, and found, as had Bovie, lowering of the coagulation temperature together with the production of a characteristic odor and yellowish color. Clark (1925, 1935) found that while proteins were altered by irradiation at other pH values, they were coagulated only near the isoelectric point. She concluded that the effects of irradiation take place in three steps: (1) a primary, mono-

molecular photodenaturation, independent of temperature; (2) a reaction between the denatured molecules and water, with a high-temperature coefficient, starting a few degrees above zero and occurring very rapidly at temperature as low as 40°, at which heat denaturation is inappreciable; and (3) flocculation of the denatured molecules as a coagulum.

The primary absorption process has been pictured as causing breaks in the molecules that enable them to be denatured at lower temperatures (Mirsky and Pauling, 1936), and as involving an opening up or unfolding of the molecule with exposure of hydrophobic groups and reactive groups such as sulfhydryl (Mirsky and Anson, 1936).

The effect of ultraviolet in rendering proteins more susceptible to heat coagulation has been suggested as an explanation of the increased heat sensitivity observed in living cells exposed to sub-lethal ultraviolet irradiation. Thus Giese and Crossman (1945) found that paramecia previously exposed to dosages of ultraviolet too small to change the rate of division were killed by thermal exposure that did not kill control organisms. Reversing the procedure (that is, exposing to heat first and then to ultraviolet) did not lead to lethal effects.

Insofar as less drastic effects that might render proteins incompatible with life are concerned, the possibilities of change in molecules as complex as proteins are so numerous, and the criteria of compatibility with continuation of biological processes so vague, that it is difficult to obtain definitive information as to the importance of such effects. One biologically significant property that can be evaluated quantitatively is the physiological activity of insulin, and it has been shown that insulin can be inactivated by exposure to far-ultraviolet radiation (Ellis and Newton, 1925). Another method of investigating the energetics of early photochemical changes is to make use of such criteria as modifications in viscosity, osmotic pressure, electrophoretic patters, and so on, as has been done by Davis, Hollaender, and Greenstein (1942). They found a marked increase in viscosity upon irradiation of horse and human serum and protein fractions, a decrease in colloid osmotic pressure, and a development of more homogeneous electrophoretic patterns. Insofar as the mechanism of irradiation effects is concerned, they concluded, as had other workers, that there is an unfolding and splitting of the molecules, followed by aggregation.

5. *Photochemistry of Purines, Pyrimidines, and Nucleic Acids*

Typical absorption spectra of purines, pyrimidines, and nucleic acids are shown in Figure 3. In the far ultraviolet range, nucleic acid absorption is analogous to that of proteins in that substantially all of the absorption is accounted for by the unsaturated heterocyclic ring structures. That is to say, nucleic acid absorption is attributable primarily to purines and pyrimidines (uracil, guanine, adenine, thymine, and cytosine), whereas the sugars and phosphoric acid contribute negligible absorption. Even in nucleoproteins, the absorption of the purine and pyrimidine nitrogenous bases is frequently sufficiently greater than that of any other molecular groupings present to account for substantially all of the absorption.

a. Purines and Pyrimidines—The photochemistry of purines and pyrimidines is of especial interest, therefore, in considering photochemical effects on nucleic acids. Unfortunately, this subject has not been studied extensively. The changes in absorption spectra that take place upon exposure of various purines, pyrimidines, and nucleic acids to far ultraviolet radiation have been investigated, and wavelength thresholds for the production of such changes have been determined (Heyroth and Loofbourow, 1931 a, b, 1933, 1934; Loofbourow and Stimson, 1940 a, b; Stimson and Loofbourow, 1941). The effective wavelength thresholds appear to be at about 2950 A., corresponding closely to the position in the spectrum at which markedly abiotic effects on cells begin, as discussed in later sections.

The absorption changes are characterized in general by an early *increase* in absorption, especially marked in the region of the short-wave absorption minimum and the long-wave steep portion of the absorption curve, followed by a later progressive decrease in absorption attributable to photodecomposition. That the early absorption changes are probably the result of molecular rearrangements was pointed out by Heyroth (1932) who found that irradiation of uracil under conditions that did not give rise to decomposition led to the production of a derivative of different oxidation-reduction potential, capable of reducing arsenophosphotungstic acid solutions.

Absorption changes following irradiation indicate rather marked differences in the stability of purines and pyrimidines to the far ultraviolet. In the above investigations, uracil exhibited a greater change in absorption after fifteen minutes than did adenine after four hours

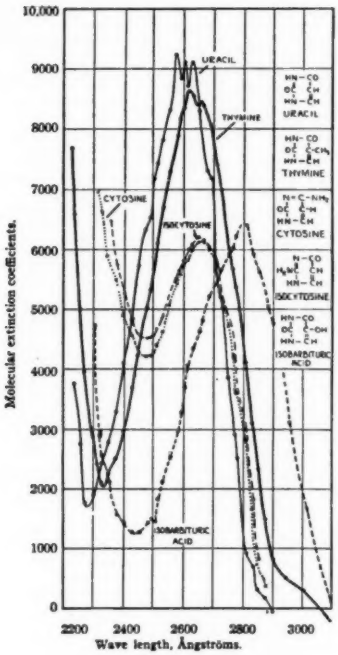

FIG. 3a.
ABSORPTION SPECTRA OF SOME PYRIMIDES (HEYROTH AND LOOFBOUROW, 1934).

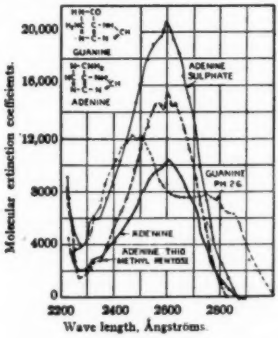

FIG. 3b.
ABSORPTION SPECTRA OF SOME PURINES (HEYROTH AND LOOFBOUROW, 1934)

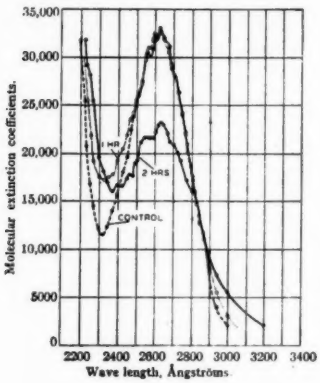

FIG. 3c.
ABSORPTION SPECTRUM OF DESOXYRIBOSE NUCLEIC ACID, SHOWING DEGRADATION BY PROLONGED IRRADIATION BY FAR ULTRAVIOLET (HEYROTH AND LOOFBOUROW, 1931a).

under similar irradiation conditions. It is therefore possible that photodecomposition of the more sensitive nitrogenous bases accounts for the major portion of the changes arising from the irradiation of nucleic acids.

b. Nucleic Acids—Ultraviolet irradiation of thymus nucleic acid leads to depolymerization and decrease in viscosity (Greenstein and Jenrette, 1941; Hollaender, Greenstein, and Jenrette, 1941-42), similar to effects produced by ionizing radiation (Sparrow and Rosenfeld, 1946). Giese (1947) irradiated nucleoprotein extracted from sea-urchin sperm, and found that irradiation progressively decreased the ability of this nucleoprotein to form fibers when added from salt solution to distilled water.

6. Photochemistry of Other Compounds of Biological Interest

A large number of other compounds of biological importance undergo photochemical change when exposed to ultraviolet radiation of appropriate wavelength (see Heyroth, 1941), and many of these substances may be involved in photobiological effects. It will be more useful to point out some general rules whereby one may deduce the likelihood of appreciable photoreactivity in various instances than to endeavor to consider all of these compounds individually.

a. General Rules Concerning Photoreactivity—From the stand-

point of energy quanta (Planck's law) in relation to the presence of absorption that might relate to chemical change (Grothus-Draper law), the visible, near ultra-violet, and far ultraviolet regions represent three distinctly different situations.

The quanta in the far ultraviolet (3000 A. to 1900 A.) are quite large (approximately 95 to 140 kilocalories per mole). Hence wavelengths in this range generally correspond to activation energies sufficient to induce chemical change, and when marked absorption occurs below about 3000 A., it may usually be assumed to be associated, at least in part, with photochemical effects.

In the near ultraviolet (4000 to 3000 A.), the quanta are large enough (about 70 to 95 kilocalories per mole) to correspond to the thermochemical bond energies of several types of bonds commonly found in organic compounds. However, the probability that absorption is associated with marked photo-reactivity is much less in this region that in the far ultraviolet. As previously discussed, photodecomposition activation energies are often higher than the thermochemical bond energies listed in Table II, and there is considerable tendency toward dissipation of excitation energy as fluorescence radiation in the near ultraviolet. Figure 4 illustrates the comparative photochemical ineffectiveness of the near ultraviolet for a specific case, that of polymerization in acraldehyde. The absorption spectrum is shown in comparison with a curve of the relative quantum yield at different wavelengths for the polymerization of this compound by radiation (Blacet, Fielding and Roof, 1937). It will be noted that the absorption is quite high in the range from 3900 to 2900 A., whereas the quantum yield within this range is low and only begins to rise markedly at about 2950 A.

In the visible region (8000 to 4000 A.), the energy quanta are still lower (about 35 to 70 kilocalories per mole), and the probability that absorption is indicative of marked photochemical reactivity is low.

To summarize: that marked absorption is indicative of corresponding photochemical reactivity is highly probable in the far ultraviolet, less probable in the near ultraviolet, and improbable in the visible.

The correlation of marked absorption in the far ultraviolet with unsaturated groupings, and particularly with conjugated double bonds, indicates that compounds known to involve such structural characteristics may be expected to be photochemically unstable in this spectral

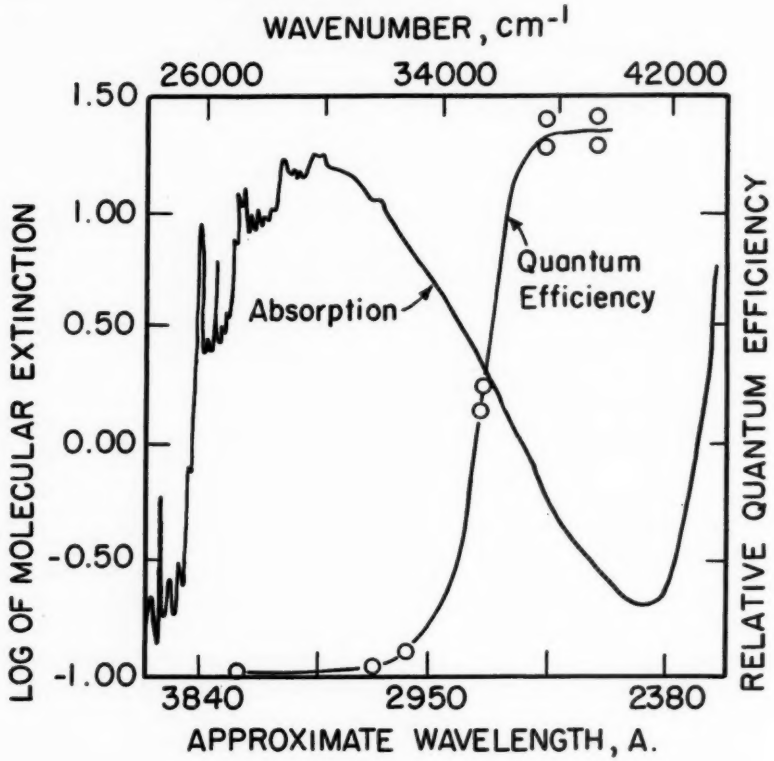

FIG. 4.
COMPARISON OF THE ABSORPTION OF ACRALDEHYDE AND THE RELATIVE QUANTUM EFFICIENCY OF ITS POLYMERIZATION BY ULTRAVIOLET RADIATION (AFTER BLACET, FIELDING, AND ROOF, 1937).

region with respect to the portions of the molecule that involve such structures.

b. Effect of Absorbing and Photoreactive Sub-Units in Complex Molecules—The probability both of absorption and of photolability in the far ultraviolet is essentially unity in those many instances in which complex molecules are known to include absorbing and photolabile sub-units. The absorption spectra and photochemical behavior of substances can, in fact, often be predicted from those of the sub-

units. Thus, the far ultraviolet spectra of diphospho- and triphospho-pyridine nucleotides (coenzymes 1 and 2) are attributable to adenine and nicotinic acid. Those of flavin-adenine dinucleotide, flavoprotein enzymes, pyridinoprotein enzymes, cocarboxylase, thiaminoprotein enzymes, and so on may be represented by the summation curves of the spectra (plotted on a molecular extinction coefficient basis) of their absorbing constituents, such as adenine, flavine, pyridine structures, thiamine, and the aromatic amino acids of proteins.

It is not surprising, on the basis of the foregoing considerations, that far-ultraviolet radiation has been found to inactivate numerous coenzymes and enzymes, including diastase (Agulhon, 1912; H son and Ashton, 1933), invertase (Joldbauer and Tappeiner, 1906), pepsin (Hussey and Thompson, 1925), saccharase (Gorbach and Pick, 1932 a, b; Svanberg, 1921), urease (Tauber, 1930; Kubowitz and Haas, 1933), amylase (Chauchard, 1913; Chauchard and Mazone, 1911; Thompson and Hussey, 1931; Zeller and Joldbauer, 1908), catalase (Koeppe, 1929, Morgulis, 1930 a, b; Stern, 1932; Waentig and Steche, 1912), emulsin (Helferich and Brieger, 1933), and tyrosinase (Narayanamurti et al., 1930). The wavelength threshold at which photoinactivation begins has been studied for many of these enzymes, and in most instances has been found to lie in the range from about 3200 to 2900 A. (Collier and Wasteneys, 1932; Agulhon, 1911; Chaucard and Dartre, 1914; Stern, 1932). The early literature on the effects of radiation on enzymes has been summarized by Schomer (1936).

c. Compilations of Absorption Spectra—Data regarding absorption spectra from which one may deduce the likelihood of photolability in accordance with the foregoing principles, have been compiled with special reference to biochemical substances by Ellinger (1937), Loofbourow (1940, 1941), and Morton (1942). Additional references to standard compilations of absorption spectra of organic compounds are given by Harrison, Lord and Loofbourow (1948).

III. Direct Effects of Ultraviolet Radiation on Cells

Agents that block or accelerate specific biochemical reactions permit one to observe the relationships of these reactions to such biological phenomena as cell division, growth, and respiration. As a consequence, there is increasing interest in their use in the study of biological problems.

The agents employed may be either chemical or physical. Among physical agents, radiant energy generally produces the most specific effects. This is particularly true in the case of ultraviolet radiation because of its direct photochemical activity. As an example, it is possible to inhibit cell division by ultraviolet radiation in certain organisms with very little apparent effect on other cellular processes. Furthermore, the inhibition appears to be attributable to photodecomposition in compounds of a particular class, namely, nucleic acids or related substances.

The use of radiant energy as a blocking or accelerating agent does not involve the addition of extraneous chemical substances to the biological system, and this may be of advantage in certain investigations. On the other hand, even ultraviolet radiation often has the disadvantage of producing less specific effects than those that can be induced by particular chemical agents. The reason for this limitation is that a very large number of substances of biological importance are labile to far ultraviolet radiation, as pointed out above.

Up to the present, the information yielded by the effects of ultraviolet regarding the role of specific biochemical substances in particular biological processes has come primarily from the application of spectroscopic techniques. The energies at different wavelengths required to block or accelerate a given biological activity are determined, and it is assumed that the curve of relative effectiveness (reciprocals of required energies) as a function of wavelength, the so-called "action spectrum", corresponds to the absorption spectrum of the substance involved in the effect.

There are limitations to this *action spectrum* method, and there are other possibilities of obtaining information about the specific relationships of biochemical substances to physiological processes. But before discussing these, we should first consider the principal effects of ultraviolet radiation on the biological activities of cells. These include inhibiting or accelerating effects on cell division, mobility, respiration, and fermentation, parthogenetic effects, production of mutations, and effects on cell-membrane permeability.

1. *Inhibition of Cell Division*

Investigations of the cell-division inhibiting, or abiotic, effects of light on microorganisms date back more than seventy years to the

period immediately following the publication of Pasteur's researches, when considerable attention was given to the influence of various chemical and physical agents on bacteria, molds, and yeasts. These early investigations indicated that near-ultraviolet radiation transmitted by glass, the effective transmission limit of which is usually about 3100 to 3400 A., and even visible radiation are capable of inhibiting cell division. Some of the early results are summarized in Table IV, and references to the considerable literature on the subject prior to 1900 will be found in the papers cited in the table.

Ward (1893, b) extended observations into the far ultraviolet. He used a quartz spectrograph to expose cultures of *B. anthracis,* spread on agar, to the spectrum from an electric arc. Subsequent incubation of the plates gave a biological spectrum of the abiotic radiation—that is to say, a kind of "action spectrum", the first to be obtained—and indicated that radiation throughout the ultraviolet as well as in the blue end of the visible spectrum was effective. Shortly thereafter, Bie (1899) reported experiments in which cultures of *B. prodigiosus* were exposed to sunlight and to carbon arc light using quartz lenses to concentrate the radiation. These experiments indicated a particularly marked effect in the far ultraviolet and initiated the beginning of a period in which the early work fell into disrepute. Many investigators came to believe that only far ultraviolet radiation had cell-division-inhibiting effects. In more recent years, the longer-wave regions have been re-explored and it has been established that near ultraviolet and visible radiation are truly bactericidal, even though their effects are of a lesser order of magnitude than those of the far ultraviolet (Bayne-Jones and Van der Lingen, 1923; Coblentz and Fulton, 1924; Hollaender, 1943; see also Hollaender, 1946). It is interesting to note that in the period in which the validity of the results of the early workers was generally denied, little or no attempt was made to reproduce the early experimental conditions; those who found no lethal effect of near ultraviolet radiation simply did not employ sufficiently large energy dosages in their investigations.

Within the past twenty years, many quantitative investigations have been carried out to establish the relation of such factors as wavelength, energy, type of organism, and so on, to the effects of ultraviolet radiation in inhibiting cell division. No attempt will be made to review this literature in detail here, since comprehensive reviews are already

TABLE IV

EARLY EVIDENCE FOR INHIBITION OF CALL DIVISION BY VISIBLE AND NEAR ULTRAVIOLET RADIATION

Light Source	Substances Interposed between Source & Organism	Organism	Exposure Time for Complete Inhibition	Most Effective Spectral Region and Method of Spectral Isolation	Reference
Sun	Glass tubes and Pasteur's medium	Mixed bacteria and molds	Few hours	Blue (filters)	Downes and Blunt (1872)
Sun	Glass tubes (bacteria exposed dry)	*B. tyrothrix*	15 days to 2 months	———	Duclaux (1885, 1887)
Sun; Gas Light	Glass tubes and broth	*B. anthracis*	2 hours	Unsuccessful attempt made to compare effects of spectral regions with a prism	Arloing (1885 a, b)
Sun	Glass tubes and nutrient medium	*B. anthracis*	30-50 hours	———	Roux (1887)
Sun	Glass dish and agar or gelatine	*B. anthracis*	6-7 hours	Blue (filters)	Ward (1892 a, b)
Sun	Glass dish only or with agar	*B. anthracis* various moulds and yeast	6-7 hours	Blue (filters)	Ward (1893 a)
Sun; Electric Arc	Quartz	*B. anthracis*	———	Blue, violet and ultraviolet (quartz spectrograph)	Ward (1893 b)
Sun	Glass tubes and bouillon	*B. anthracis*	6-7 hours	———	Ward (1894)

available (Duggar, 1936; Giese, 1942, 1945, 1947; Hollaender, 1942, 1946). Some of the more important conclusions only will be summarized.

a. Threshold Wavelengths—With the exceptions noted above, the early workers had either employed sunlight as a source, with an effective short-wave limit of about 2950-3000 A., or had interposed glass between the irradiated organisms and the source. Since ordinary glass transmits very little radiation below about 3200 A., there was no opportunity in the early work to observe the particularly marked effects of far ultraviolet radiation on cell division.

As soon as the region below 3000 A. began to be explored, it became apparent that abiotic effects increase very markedly at about 3000-2900 A. The existence of a wavelength threshold at which inhibition of cell division increases abruptly toward shorter wavelengths may be shown most simply by following Ward's technique of exposing a smear of bacteria to the spectrum formed by a quartz spectrograph and subsequently incubating the smear until growth occurs in the regions in which there has been no abiotic effect. Figure 5 shows such an abiotic spectrogram taken by Browning and Russ (1917-19) with a Hilger quartz spectrograph and a tungsten arc source. The striking change in effect in the 3000-2900 A. region may be seen quite easily in this plate.

Table V summarizes the approximate wavelength thresholds at which

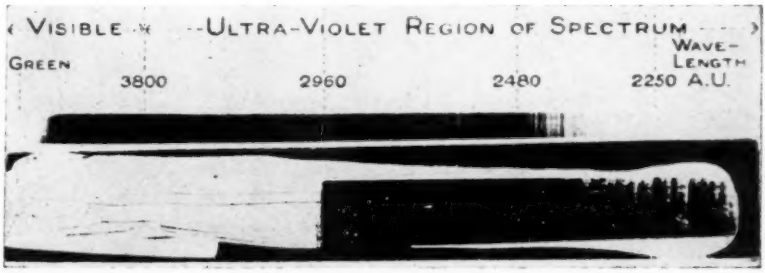

FIG. 5.

"ABIOTIC SPECTROGRAM" MADE BY EXPOSING A SLIDE COATED WITH AGAR AND INOCULATED WITH *S. aureus* TO THE SPECTRUM OF A TUNGSTEN ARC IN A QUARTZ SPECTROGRAPH FOR 3½ HRS. AND SUBSEQUENTLY INCUBATING THE PLATE. THE DARK AREA REPRESENTS THE REGION IN WHICH THE ORGANISMS FAILED TO GROW. A PHOTOGRAPH OF THE TUNGSTEN ARC SPECTRUM IS SHOWN AT THE TOP OF THE SLIDE (BROWNING AND RUSS, 1917-19).

TABLE V

APPROXIMATE WAVELENGTH THRESHOLD AT WHICH INHIBITION OF CELL DIVISION INCREASES ABRUPTLY TOWARD SHORTER WAVELENGTHS

Organism	Effect	Wavelength Threshold, A.	Method	Reference
S. aureus *B. coli*	50% killing	3020	Monochromator	Gates (1930)
Paramecium	Vesicle formation in 75%	3130 to 3020	Monochromator	Weinstein (1930)
	Lethal action	3020	Monochromator	Giese and Leighton (1935)
B. prodigiosus	Complete inhibition of division	3000 to 2800	Filters	Bang (1905)
Sea urchin eggs	Doubling of time of 3rd cleavage	3020	Monochromator	Geise (1938)
Bacteria (7 species)	Complete inhibition of division	3020	Abiotic Spectrogram	Burger (1928)
Bacteria (7 species)	Complete inhibition of division	3290	Abiotic Spectrogram	Barnard and Morgan (1903)
Bacteria (12 species)	Complete inhibition of division	3020	Filters	Cernovodeanu and Henri (1910)
B. typhosus	Complete inhibition of division	2970	Abiotic Spectrogram	Newcomer (1917)
S. cerevisiae	Complete inhibition of division	2890	Filters	Loofbourow and Cameron (1933)
S. aureus *S. albus* *B. coli*	Complete inhibition of division	2960	Abiotic Spectrogram	Bayne-Jones and Van der Lingen (1923)
B. coli	Complete inhibition of division	2800 to 3050	Filters	Coblentz and Fulton (1924)
Bacteria (4 species)	Complete inhibition of division	2960 to 3000	Abiotic Spectrogram	Browning and Russ (1918)

abrupt increases in the cell-division-inhibiting effect have been found for various organisms by different workers. It will be noted that the threshold is in all instances approximately the same as the short-wave limit of the sun's spectrum at the earth's surface. This correlation was interpreted quite early as an example of adaptation of organisms. Thus, Cernovodeanu and Henri (1910) stated "it seems then that there is in this fact a law of adaptation of living organisms to the sun's light; the protoplasm of all living beings is altered by the extreme ultraviolet rays". More broadly, one can say that the existence of intense solar radiation at wavelengths longer than about 2950 A. is incompatible with abundance on the earth's surface of molecular structures that are photochemically labile at such wavelengths. *That is to say, the equilibrium between radiation and matter at the earth's surface favors the abundance of molecules with photodecomposition activation energies greater than that represented by the wavelength limit of the solar spectrum.*

b. The Reciprocity Law—The reciprocity law (also known as the Bunsen-Roscoe law) states that the effect of exposure to radiation is a function of the total energy and is independent of the time and intensity when these are varied in reciprocal proportions so as to result in the same total energy. In symbols:

$$Q = KE = KIt, \tag{3}$$

where Q is a standard effect (for example, killing of 50 per cent of irradiated organisms), K is a constant, E is the total energy dosage per unit area, I is the intensity and t is the time of exposure. In a typical case, E may be expressed in ergs per mm.^{2}, I in ergs per mm.^{2} per sec., and t in secs.

A little reflection will show that this law can be valid for photobiological effects only within restricted limits of variation of t. Thus, if t is long enough so that the progress of events other than the photochemical action of the radiation has an influence on the biological effect, the outcome will be modified accordingly. An energy density of 100 ergs per mm.^{2} at 2600 A. may suffice to kill 50 per cent of a unicellular layer of a particular microorganism if administered in one minute, whereas this same energy density would obviously not have the same biological effect if administered at so low an intensity level that exposure for one year would be required for the same dosage.

During a year the organisms would, in the normal course of events, either have died of other causes or have multiplied manifold so that no individual organism as such would be exposed to a lethal dosage.

The reciprocity law is therefore meaningful only when restricted to times so short that other kinetic activities do not modify the course of the reaction.

Investigations of the reciprocity law in relation to the cell-division-inhibiting effect of far-ultraviolet radiation have usually been restricted to times of exposure short in relation to the life cycle of the organisms studied. Under these conditions, the law has been found to be approximately valid. This result is reasonable, since there is no *a priori* reason why a given energy dosage should not yield a given photochemical and corresponding photobiological effect, regardless of the time in which it is administered, provided the time is so short that other kinetic activities do not modify the effect. Measurable departures from the reciprocity law have, however, been found by several workers even for ranges of exposure up to a few minutes.

Using times of exposure of 4 to 300 sec., and distances of 10 to 60 cm., Cernovodeanu and Henri (1910) found that the time required for complete killing of *B. coli* increased more rapidly than the square of the distance from a 100 volt quartz mercury arc source*, the times being in the ratios of 1, 5, 45, and 75 for squares of distances in the ratios 1, 4, 16, and 36. Somewhat inconsistent results were obtained with another source. With a quartz mercury arc screened by mica filters, Coblentz and Fulton (1924) investigated the relation of time to intensity for equal killing effects on *B. coli*, the times ranging from 10 to 1250 secs. When the intensity was reduced to 0.1 or 0.02, the exposure time had to be increased by 12.5 to 14.0x and 68 to 75x respectively for the same photobiological effect. Gates (1929 b) found that the exposure time had to be increased in somewhat greater than inverse proportion for equal killing of *S. aureus* by 2660 A. radiation when the intensity was decreased from 21.6 to 5.6 ergs per mm.^{2} per sec., but the departure from the reciprocity law was progressively less as the percent of cells killed was increased. The exposure times

*It is not valid, of course, to assume that the inverse square law holds strictly for a case of this sort. Deviations from the law begin to be appreciable when the distance from source to receiver is less than about 25 times the largest dimension of source cross section. It is reasonable to assume approximate applicability of the inverse square law in this instance, however.

involved varied from about ½ to 60 secs. Rentschler, Nagy, and Mouromseff (1941) claim that the reciprocity law is valid for the lethal action of 2537 A. radiation on *E. coli* over a range of times from a few microseconds to several hours. These results are at variance with those previously cited, and it is difficult to deduce from the limited data they present what factors may account for the discrepancy. Finally, Rentschler and Giese (1941) found that microsecond flashes of high-intensity ultraviolet radiation from a quartz mercury vapor resonance lamp retarded the division of *Paramecium* to the same extent as ten second exposures at correspondingly reduced intensities. In this instance, the range of times was ten millionfold, but the maximum exposure time was quite short.

c. The Intermittency Effect—The relative photobiological effects of intermittent and continuous exposures to the same total radiant energy have been studied by several investigators cited in connection with the reciprocity law. In general, intermittent exposures are found to have substantially the same effects as continuous exposures. On theoretical grounds, this result is consistent with a mechanism in which photobiological effects are the direct result of photochemical processes.

d. Bactericidal Effect as a Function of Wavelength—Bang (1905) determined the relative killing times required for *B. prodigiosus* at various wavelengths in the far ultraviolet, but it remained for others to carry out such experiments with carefully controlled energy dosages.

In making quantitative measurements, a quartz monochromator of high transmission is usually employed. Unless relative values only are given, the dosage is usually expressed in terms of total incident energy in ergs/cm.^{2} or ergs/mm.^{2} required to produce a given biological effect (such as death of 50% of the organisms). The dosage is ordinarily controlled by varying the time of exposure. It is assumed that the reciprocity law holds for the different values of I and t employed. As pointed out above, various workers have observed appreciable deviations from the reciprocity law for exposure times differing by minutes; one should therefore consider the possible influence of this effect when evaluating the experimental results reported in the literature.

Investigations of the relation of wavelength and energy to inhibition of cell division have now been made by many workers, among the

earliest of whom were Gates (1929 a, b, 1930), Weinstein (1930), Wyckoff (1932), Oster (1934-35 a, b, c) and Oster and Arnold (1934-35). References to the more recent literature will be found in the reviews previously cited. In general, there is a maximum effectiveness at about 2500-2600A., an abrupt decline in effectiveness at about 2900-3000 A., and a progressively lower effectiveness from this point through the near ultraviolet into the visible. Some, but not all, workers have also observed a second minimum at about 2300-2400 A., with a progressive rise in effectiveness toward shorter wavelengths.

Data for the relative effectiveness of different wavelengths in inhibiting *E. coli* colony formation are representative, and values obtained by Gates (1930) and Luckiesh and co-workers (1946) are given in Table VI.

TABLE VI

ENERGIES AND RELATIVE EFFECTIVENESS (IN TERMS OF RECIPROCALS OF INCIDENT ENERGIES OR QUANTA TO PRODUCE THE EFFECT) OF DIFFERENT WAVELENGTHS FOR INHIBITION OF COLONY FORMATION BY E. COLI

Wavelength in A.	Incident Energy for 50% killing, in ergs/mm. (Gates, 1929)	Relative Effectiveness			
		Gates (1929) (50% killing)		Luckiesh (1946) (Percent killing not stated.)	
		Energy Basis	Quantum Basis	Energy Basis	Quantum Basis
2259				0.33	0.37
2302	12.5	0.64	0.745	0.40	0.44
2353				0.50	0.54
2379	22.2	0.36	0.406	0.55	0.59
2399				0.62	0.66
2446				0.73	0.76
2483	10.8	0.74	0.794	0.84	0.86
2537				1.00	1.00
2576				1.00	0.99
2654				0.96	0.92
2675	8.0	1.0	1.00	0.90	0.85
2700				0.87	0.82
2753				0.72	0.66
2804	18.3	0.44	0.42	0.57	0.52
2894				0.31	0.27
2925				0.23	0.20
2967				0.13	0.11
3022	245	0.033	0.029	0.045	0.038
3129				0.008	0.0065
3341				0.0013	
3654				0.00023	
4047				0.00009	
4358				0.000058	
5461				0.000031	
5780				0.000028	
6830				0.000020	

e. Percentage of Cells Inhibited as a Function of Incident Monochromatic Energy—At any particular wavelength, the percentage of cells inhibited from dividing in a large population is a function of the energy dosage. At very low energies no organisms are inhibited from dividing, according to the results of most workers; that is to say, there is a threshold energy dosage below which no effect takes place (see Gates, 1929 b). Above the threshold, the percentage of inhibited cells increases rapidly at first and then more slowly. If the logarithm of the percent of uninhibited cells is plotted as a function of the energy dosage (Wyckoff, 1932; Oster, 1934-35 a, b, c; Hollaender, 1942; Luckiesh, 1946), or the percentage of inhibited cells is plotted as a function of the logarithm of the energy dosage (Gates, 1929 a, b, 1930) the curve may approximate a straight line over a considerable range of values. This relationship may be interpreted in various ways (see Gates, 1929 b), one interpretation being that the photochemical process involved is a monomolecular reaction. It should be remembered that the different energy dosages in such experiments are usually obtained by varying the time of exposure, and that the possible influence of departure from the reciprocity law should therefore be taken into account in evaluating the experimental results.

If one assumes the primary process to be a monomolecular reaction in which the absorption of one effective quantum is sufficient to inhibit division of a single cell, one may then compute from the average number of quanta per cell required for inhibition that the vulnerable portion of the cell must be quite small. From experimental data indicating that about 4.2×10^{6} quanta are required per cell at 2699 A., on the average, for lethal effect, Wyckoff (1932) computed the sensitive volume for *B. coli* to be 9.38×10^{-20}cc., the order of magnitude of the size of a single protein molecule of molecular weight 120,000. This theory of lethal action, known as the "target hit theory" has been but little applied to ultraviolet radiation, but has received extensive consideration by investigators of ionizing radiation. A detailed discussion of the target hit theory is given by Lea (1947), and we shall not consider the pros and cons here, but it should be pointed out that it appears more applicable to the explanation of some of the effects of ionizing radiation than to effects of ultraviolet.

Since there is no clear-cut end point at which cell division is completely inhibited in all organisms, many workers express their data in

terms of the dosages that result in a particular percentage of uninhibited organisms. The x-ray workers ordinarily use 37 per cent survivors, or unaffected cells, as the end point. This follows from the approximately logarithmic relationship between percentage of survivors and energy dosage. If N_o is the total number of cells, N the number of survivors at time t, I the intensity of the radiation, and k an appropriate constant, then the expression for the fraction of survivors as a function of dosage is as follows, provided the survival curves are logarithmic and the reciprocity law holds:

$$\frac{N}{N_o} = e^{-kIt} \tag{4}$$

When $kIt = 1$, the value of N/N_o is $1/2.718$, or 0.368, that is to say, about 37%. This is a convenient standard biological effect, for if $kIt = 1$, then $k = 1/It$ and computations are correspondingly simplified. Workers with ultraviolet radiation usually use other standard biological effects; Gates (1929 a, b, 1930), for example, expressed his data in terms of 50 per cent survival ratios.

If one computes the number of quanta per cell required for almost any biological effect of ultraviolet radiation, it is generally true that the value is extremely large. This implies that a very large number of molecules in the cell must undergo photochemical change in order for grossly observed biological effects to take place, that the fundamental photochemical processes involved have a low quantum efficiency, or both; an alternative interpretation is the target hit theory as indicated above.

f. Relative Sensitivity of Different Organisms—It is difficult to obtain reliable data on the relative sensitivities of different organisms because various workers have used different conditions of irradiation; but approximate values can be compiled from the literature. Thus, comparative data on the incident energies at 2537 A. required for 90 per cent killing have been tabulated for a large number of microorganisms by Hollaender (1942). Typical values in ergs/mm.^{2} are as follows: *B. anthracis*, 452; *E. coli*, 300; *B. subtilis spores*, 1200; *S. albus*, 184 to 300; and *S. aureus*, 218 to 495. Giese (1945, 1947) gives compilations of energies at different wavelengths required to produce various biological effects. As an example, at 2537 A. a dosage of

about 25,000 ergs/mm.^{2} is required for complete suppression of cleavage in sea urchin eggs at prophase (Giese, 1947).

g. Abiotic Effects on Other Biological Systems—While cell-division-inhibiting and lethal effects have been investigated most extensively with microorganisms, many other systems have also been studied. Lethal effects on tissue cultures, following in general somewhat the same pattern as that described above, have been reviewed by Mayer (1939). Such phenomena as inhibition of division of sea urchin eggs. lethal effects on viruses and bacteriophage, and similar inhibiting or lethal actions on other biological systems are summarized in the reviews previously cited.

2. Other Direct Effects on Cells.

In many respects, cell-division-inhibiting and lethal effects are less interesting, and, perhaps less significant from the standpoint of elucidating biological phenomena than other effects of ultraviolet radiation on cells. It will be practicable to consider these other effects only very briefly, however, limiting the discussion to illustrative examples.

a. Production of Mutations; Effects on Chromosomes and Mitosis—It is well established that far ultraviolet radiation is capable of inducing mutations under circumstances in which it is not prevented by absorption from reaching genetically active areas. Among early workers, Mme. V. Henri (1914) observed ultraviolet-induced mutations in *anthrax bacilli.* Extensive investigations of such effects in maize have been carried out by Stadler and his co-workers (Stadler and Sprague, 1936; Stadler, 1939). Hollaender and co-workers have studied mutations induced by ultraviolet in dermatophytes (Emmons and Hollaender, 1939, 1945) and fungi (Hollaender, Raper, and Coghill, 1945; Raper, Coghill and Hollaender, 1945; Hollaender and Emmons, 1941; Hollaender, Sansome, Zimmer, and Demerec, 1945). In many of these and other studies, the wavelength dependence of the effects has been emphasized, as discussed in the subsequent sections on action spectra. Among the most interesting investigations are those in which ultraviolet radiation has been employed to produce mutant forms having special biochemical requirements as in the work of Tatum and Beadle (Tatum, 1944; Tatum and Beadle, 1945).

Swanson (1940, 1942) developed a pollen-tube technique, using *Tradescantia,* for observing ultraviolet effects on chromosomes. At

2537 A., the number of deletions he observed was, within considerable limits, proportional to the dosage. The incident energy requirement for 5 per cent deletions at 2537 A. was about 3000 ergs/mm.^{2} Marked shortening of the chromosomes was observed in metaphase following treatment at prophase with 2537 A. radiation, but not with 2967-3022 A. radiation.

In investigation of the effects of 2537 A. radiation on mitosis in the grasshopper neuroblast, Carlson and Hollaender (1945) found an intensity effect (failure of the reciprocity law) at high, but not at low, energy doses, from which they deduced that both primary and secondary effects are involved in inhibition of mitosis by ultraviolet.

b. Parthogenetic Effects—Unfertilized sea urchin eggs treated with ultraviolet radiation and subsequently with hypotonic salt solution exhibit cleavages (Loeb, 1914; Lillie and Baskervill, 1922). Cleavages can also be induced by ultraviolet irradiation only, and it has been suggested from the wavelength dependence that the substance involved is a protein (Hollaender, 1938). At 2260 A., incident energies of about 2.3 to 4.2 ergs per egg are required to activate 80 per cent of the eggs.

c. Effects on Respiration and Fermentation—Early work on the effects of ultraviolet on the respiration of cells has been reviewed by Wynd and Reynolds (1935). Many investigators found only depressing effects, but a few (for example, Suranyi and Vermes, 1929; Tang, 1936) observed increases in oxygen uptake. Giese (1942) investigated this problem for yeast and found that the endogenous respiration is increased by ultraviolet radiation but that the exogenous respiration exhibits only depression. The effect could be observed only with yeast of low endogenous respiration irradiated with nutrient absent or present only in small amounts.

Many workers have reported that ultraviolet increases the rate of fermentation of yeast (for example, de Fazi and de Fazi, 1921; Owen, 1933), whereas others have noted only depressing effects (Tanner and Byerly, 1934). The differences in results may be attributable in part to unlike experimental conditions; in some instances the conditions appear to have been such that effects of radiation-damaged cells on undamaged cells may have played a part in the phenomena observed (see Part V).

d. Stimulation of Cell Division—Numerous investigators have reported observations of acceleration of the rate of cell division of

organisms following exposure to sublethal dosages of far ultraviolet radiation. The literature has been reviewed recently by Giese (1947). Apparent accelerating effects are sometimes attributable to such factors as the additional nutrient medium available to surviving colonies in areas of bacterial surface cultures that have been irradiated sufficiently to kill an appreciable proportion of the cells. But even when all such explanations are taken into account, there appears to be a real acceleration of the division rate under particular circumstances of irradiation and with particular organisms (Coblentz and Fulton, 1924; Meier, 1939; Meier-Chase, 1941; Hollaender and Duggar, 1938).

The mechanism of these accelerating effects is not clearly understood. As Giese (1947) points out, such stimulative effects as those found by Meier for algae, in which the accelerated rate of division persists through successive generations, may very likely be attributable to radiation-induced mutations. In other instances, the effects appear to be confined to cells directly exposed to radiation, and are somewhat analogous to stimulating effects observed with other lethal agents, such as toxic substances, at low dosage levels. In still other instances, the effects may possibly be attributable to products from radiation-damaged cells that accelerate the division rate of undamaged cells, as discussed in Section V.

e. Carcinogenic Effects—The carcinogenic effects of prolonged dosages of ultraviolet radiation, as for example in the exposure of the skin of animals to the ultraviolet radiation of sunlight, have long been known (see Blum, 1940), and have been investigated with considerable care in recent years (see Hollaender, 1946, for a brief summary of the recent literature). The most effective incident wavelengths appear to be in the neighborhood of 3000 A. (Blum, 1943). Since investigations of the effect involve the exposure of intact animals to radiation, the relative effectiveness of different incident wavelength regions is influenced not only by the wavelength dependence of the photochemical reactions responsible for carcinogenesis, but also by the absorption of radiation in tissue between the surface and the site of carcinogenesis.

Bain and Rush (1943) found that long exposures at low intensities in the 2800-3400 A. region were more effective than the same energy dosages at high intensity levels. Blum, Grady, and Kirby-Smith (1942) investigated the effects of intermittent exposures, and found that under their experimental conditions, the time of induction of tumors by ultra-

violet decreased progressively when the same dosage was given in five or seven exposures instead of one exposure per week. The departures from the reciprocity law and the effects of intermittent exposures indicate that a complex interaction of photochemical and physiological processes is involved.

f. Other Effects—The above examples of direct biological effects of ultraviolet radiation on cells are typical, but there are many others. Thus, far ultraviolet radiation has been found to inhibit neural-fold formation in the chick and to retard excystment of *Colpoda*. Sub-lethal doses of far ultraviolet radiation have been shown to give rise to increased membrane permeability in certain cells and, under particular circumstances, to changes in the morphology of cells. These and many other effects are summarized in the general reviews previously listed.

IV. Specific Reactions Involved in the Effects of Ultraviolet Radiation on Cells

It will be evident from the preceding discussion that the effects of far ultraviolet radiation on cells are many and varied, as might be expected from the large number of essential biochemical substances that are photolabile in the far ultraviolet. The usual pattern of the effects is one of markedly altered cellular metabolism or of biological disfunction. It is generally accepted that the primary processes responsible for the effects are photochemical changes in cellular constituents, and that indirect effects arising from photochemical change in the extracellular milieu are of secondary or negligible importance in comparison with direct effects on cells under most experimental conditions. In this section, we shall first examine the conditions under which effects on the milieu may be considered to be unimportant, after which we shall discuss evidence as to the specific photochemical effects that appear to be responsible for some of the photobiological responses previously described.

1. Photochemical Changes in the Extracellular Milieu

The possibility that changes in irradiated cells may arise from photochemical effects in the extracellular medium was investigated by even the earliest workers (Downes and Blunt, 1877) and has been reinvestigated repeatedly since that time. If the variations in experimental conditions employed by different workers are taken into account,

one finds in these experiments consistent evidence for the following conclusions: (1) under certain conditions of irradiation, products that are either toxic or less favorable than their precursors to normal cell activity may be formed in the environmental milieu, and (2) under most experimental conditions the effects of such products are inconsequential in comparison with the effects of direct photochemical changes within the cells.

The photochemical products to which milieu effects have usually been attributed are: (1) hydrogen peroxide formed in water or aqueous solutions (Bedford, 1927), (2) photodecomposition products in agar or other nutrient media (Blank and Arnold, 1935; Baumgartner, 1936; Kersten and Dwight, 1937) and (3) ozone formed in atmospheres containing oxygen. We shall consider each of these in turn.

a. Formation of Hydrogen Peroxide—Pure water is extraordinarily transparent to ultraviolet of wavelength greater than about 1900 A., and on that account alone would be expected to be but little affected by far-ultraviolet radiation. After exposure to energies of full ultraviolet of considerably greater orders of magnitude than those involved in photobiological experiments, detectable amounts of hydrogen peroxide have been found in water (Kernbaum, 1909). The reaction is brought about by radiation of wavelengths below 1900 A. in the presence of dissolved oxygen and possibly other contaminants (see Heyroth, 1941, p. 339). Investigations of the photochemistry of the production of hydrogen peroxide in gaseous hydrogen and oxygen indicate, both on the basis of theory and experiment, that the reaction is essentially confined to the region below about 1900 A., whereas decomposition of hydrogen peroxide occurs throughout the far ultraviolet (see Noyes and Leighton, 1941; Heyroth, 1941). At wavelengths longer than about 2000 A. in the ultraviolet, one would therefore not expect to find photobiological effects attributable to the formation of hydrogen peroxide in aqueous media.

The negligible role of water is one characteristic that distinguishes photobiological effects in the far ultraviolet from the radiobiological effects of ionizing radiation. X-rays, gamma rays, and other kinds of ionizing radiation may form appreciable amounts of hydrogen peroxide in water, as well as large numbers of highly reactive ion pairs.

b. Photodecomposition in Nutrient Media—Photodecomposition of carbohydrates may result in unfavorable changes in the pH of nu-

trient media (Baumgartner, 1936), and if photolabile growth factors, such as thiamine or riboflavine, are present, these may be degraded to inactive substances upon exposure to far ultraviolet radiation for sufficiently long periods of time. Depending upon the composition of the medium and the conditions of irradiation, products that are toxic for the organism under study may or may not be formed in detectable quantities. Coblentz and Fulton (1924) found, for example, that irradiation of culture media for periods considerably longer than those required to kill surface innocula of *B. coli* rendered the media unable to support growth. In those special types of experiments that involve the simultaneous irradiation of organisms and nutrient media, however, changes in the medium are usually evaluated by suitable control procedures, and the experimental conditions are usually such that effects arising from changes in the medium are negligible in comparison with those attributable to changes induced directly in the cells (see, for example, Gates, 1929 a).

c. Formation of Ozone—If the medium external to the cells is air, the principal constituent that must be considered from the standpoint of photochemical effects in the far ultraviolet is oxygen. Nitrogen, hydrogen, and the inert gases have negligible absorption in this region and are quite stable to irradiation even in the Schumann range (see Harrison, Lord, and Loofbourow, 1948, Chapter XIX). Oxygen exhibits weak absorption below about 2400 A., and has a region of more marked absorption below about 1750 A.

The formation of ozone by the ultraviolet irradiation of oxygen has been observed at wavelengths as long as 2537 A., but only at pressures of about 100 atmospheres or more (Warburg, 1912, 1914). In the region below about 2000 A., appreciable ozone is formed from oxygen even at atmospheric pressures. It is common experience, for example, that the odor of ozone can be detected in the vicinity of a bare quartz mercury arc, especially when the burner is new and the short-wave ultraviolet output is comparatively high. The effect is attributable primarily to the 1851 A. mercury line (see Noyes and Leighton, 1941; Heyroth, 1941). Hence when irradiation is carried out in the presence of air with a bare quartz mercury arc, ozone may contribute to the effects observed unless precautions are taken to exclude this possibility.

One may assume that there is little likelihood of ozone contributing a major portion of the effect if (1) the radiation reaching the organisms

and their surrounding medium is limited to wavelengths longer than about 2100 A. (for example, by filters or a monochromator) and (2) the possibility of transport of ozone from the vicinity of the source itself so as to come in contact with the organisms is excluded (for example, by placing the organisms in a closed system).

d. Conclusions Regarding Milieu Effects—The above photochemical considerations indicate that appreciable effects of photochemical products formed directly in the extracellular milieu are to be expected only under special conditions of irradiation. Nevertheless, in any given investigation, milieu effects should be ruled out by adequate control procedures before it is assumed that direct photochemical changes in cells are entirely responsible for the photobiological phenomena observed. Of possible perturbing factors, ozone is perhaps most troublesome, and special care should be used to exclude its influence.

In general, suitable control procedures have demonstrated that direct effects on cells are primarily responsible for the various photobiological effects previously described, under usual experimental conditions. Thus, cell-division-inhibiting effects comparable to those discussed earlier have been obtained upon irradiation in the absence, as well as in the presence, of water or nutrient media, and in inert atmospheres as well as in air.

2. *Specific Types of Reactions Indicated by Action Spectra*

a. Action-Spectrum Techniques—The determination of precisely what takes place in a cell when its functions are disrupted by ultraviolet radiation is a difficult problem, especially in view of the present lack of data regarding the photochemistry of biochemical substances. As indicated previously, the general approach up to this time has been to make use of *action spectra* as a means of obtaining evidence regarding the cellular substances involved. To obtain an action spectrum, one determines the incident energies required at different wavelengths to produce a specific biological effect. The reciprocals of these incident energies are then plotted as a curve of relative spectral effectiveness, and it is assumed that this curve corresponds more or less closely to the absorption spectrum of the substance responsible for the effect.

An analogous method was early employed in chemical studies to identify absorbing structures at low concentration by determining

their photochemical modification as a function of wavelength (see, for example, Warburg, 1927). Application to photobiological investigations is fundamentally more difficult because of the greater complexity of biological systems. Under suitable conditions, however, the method yields useful indications of the specific chemical substances responsible for photobiological effects.

As a typical example, let us consider the data for the relative spectral effectiveness of ultraviolet radiation in inhibiting colony formation in *E. coli* as given in Table VI. These data are plotted in Fig. 6, in comparison with the relative absorption of ribose nucleic acid at different wavelengths. The resemblance of the relative spectral effectiveness curve to the relative absorption of nucleic acid is quite striking, and it seems reasonable to assume, as was done in early investigations of this type (Gates, 1930; Heyroth and Loofbourow, 1934) that either nucleic acids, or substances having similar absorption (for example, purines or pyrimidines), are involved in this photobiological effect.

b. Summary of Action-Spectrum Studies—Action spectra have now been determined for a considerable number of the biological effects of ultraviolet radiation. Giese (1945) has reviewed and correlated this work. He divides action spectra into seven types, as follows:

(1) Those with maxima at about 2600 A. and below 2400 A. and a minimum between these maxima. Attributed to nucleoproteins. Characteristic of lethal effects on cells and of production of mutations.

(2) Those with maxima at about 2800 and below 2500 A. and with minima between 2500 and 2600 A. Attributed to cytoplasmic proteins containing aromatic amino acids. Characteristic of such effects as immobilization and vesiculation of *paramecia*, retardation of excystment of *colpoda*, prevention of hatching of nematode eggs, etc.

(3) Those in which the effect begins to be noticeable below 2650 A. and rises progressively toward shorter wavelengths. Attributed to cytoplasmic proteins without appreciable content of aromatic amino acids. Characteristic of activation of sea urchin eggs.

(4) Those in which the effect begins to be noticeable in the neighborhood of 2800 A. and rises progressively toward shorter wavelengths. Attributed either to cytoplasmic proteins with inappreciable aromatic amino acid content or to lipids. Characteristic of the hemolyzing effect of ultraviolet radiation.

(5) Those with maxima at 2970 and below 2500 and a minimum

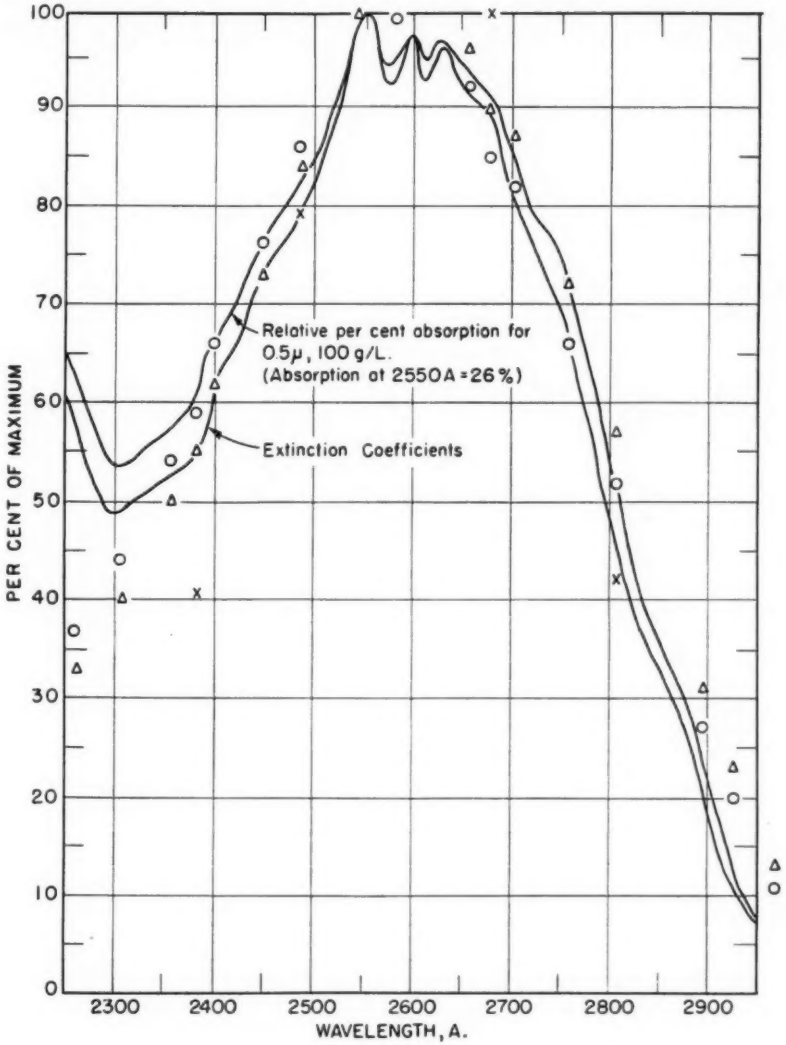

FIG. 6.

RELATIVE ABIOTIC EFFECT OF ULTRAVIOLET ON *E. coli* COMPARED WITH RELATIVE ABSORPTION OF RIBOSE NUCLEIC ACID. CIRCLES AND TRIANGLES REPRESENT RESPECTIVELY RECIPROCALS OF QUANTA AND RECIPROCALS OF ENERGIES FOR ABIOTIC EFFECT FROM DATA OF LUKIESH ET AL. CROSSES REPRESENT RECIPROCALS OF QUANTA FROM DATA OF GATES (SEE TABLE VI).

at about 2800 A. Possibly attributable in part to absorption by proteins in surface epithelium. Characteristic of the production of erythema and of secondary tanning following erythema.

(6) Those with a maximum at about 3400-3500 A. decreasing in effectiveness on either side of this point to negligible effectiveness at about 2900 A. and 4300 A. Attributable to a precursor of oxidized melanin. Characteristic of primary tanning reactions, not preceded by erythema.

(7) Those with maxima near 2600 A. and 2800 A. and minima at 2600-2700 A. and below 2500 A. Attributed to sterols. Characteristic of inhibition of neural-fold development in the chick.

3. *Theoretical Basis of Action Spectra*

Since action spectra are employed extensively for deriving conclusions as to the chemical factors involved in the blocking or acceleration of specific biological processes, it is important to consider the theoretical basis upon which their interpretation rests. Little attention has been given to the quantitative aspects of the theory in biological publications. As a consequence, the conditions that limit the reliability of the information obtainable by action spectra are not well defined in the literature. It would be out of place to attempt a rigorous treatment of the problem here; the following analysis will suffice to define the important parameters and to indicate their relative influence on experimental results.

a. Fundamental Assumptions—Consider a specific case in which one determines the incident energy densities, in ergs per mm.^{2}, at various wavelengths required to kill 50 per cent of a single-cell layer of microorganisms spread upon the surface of a nutrient medium (as in the experiments of Gates, 1929 a, b). A standard biological effect is produced in each instance, and the variables observed are wavelength, λ, intensity, I, and time of exposure, t. If it is assumed that the standard biological effect always corresponds to photochemical change in N molecules of an essential substance, on the average, per cell, then N is the same for all wavelengths. But

$$N = \gamma \frac{E_a}{h\nu} = \gamma \frac{E_a \lambda}{hc} \tag{5}$$

where γ is the quantum efficiency of the photochemical reaction (that

is to say, the number of molecules changed per quantum of energy absorbed), E_a is the energy absorbed by the essential substance in an average cell, h is Planck's quantum constant, ν the frequency of the radiation, and c the velocity of light. For production of the standard biological effect at two wavelengths, λ' and λ'',

$$\gamma' \frac{E'_a\lambda'}{hc} = \gamma'' \frac{E''_a\lambda''}{hc}$$

$$\text{or } \gamma' E'_a\lambda' = \gamma'' E''_a\lambda'' \qquad (6)$$

b. Effect of Scattering and Absorption by Inactive Substances—In general, the energy E_a absorbed by the essential substance in an average cell will be but a fraction of the energy E_o incident upon the cell. Part of the incident energy will be dissipated by reflections and scattering, part by transmission through the cell, and part by absorption by substances not related to the biological effect. We must now set up an expression relating E_o to E_a at various wavelengths, in which these other energy dissipating factors are taken into account. Let us consider a small element of volume, dV, in any one of the irradiated cells (Fig. 7), representing the path followed by a beam of the incident radiation of cross sectional area dA. The analysis to be used in determining the effect on this particular element of volume applies to any other element in the same cell or in any other cell in the irradiated population, and the conclusions may, therefore, be generalized to apply to the irradiated population as a whole. Within dV, photochemical change in n molecules of the essential substance (where n is a fraction of N) may be assumed to correspond to the standard biological effect. Let I_o be the intensity of radiation, for example, in ergs per mm.^{2} per sec., incident on the surface dA. Suppose the essential substance is confined to a limited volume in the cell (Fig. 7). Before the radiation reaches this region it will, in general, be reduced in intensity to a new value, I_1 as a result of reflections and of absorption by biologically inactive substances. If the path length from the surface of the cell to the region of the essential substance is x_1, the absorption coefficients of inactive absorbing substances distributed evenly throughout elements of this path are a_1, a_2, a_3, . . . etc., and the fractions of the radiation transmitted at various surfaces of reflection are T_1, T_2, T_3, . . . etc., then I_1 is related to I_o as follows:

$$I_1 = I_o\,(T_1\,T_2\,T_3\ldots)\,(\epsilon^{-a_1x_1}\,\epsilon^{-a_2x_1}\,\epsilon^{-a_3x_1}) \qquad (7)$$

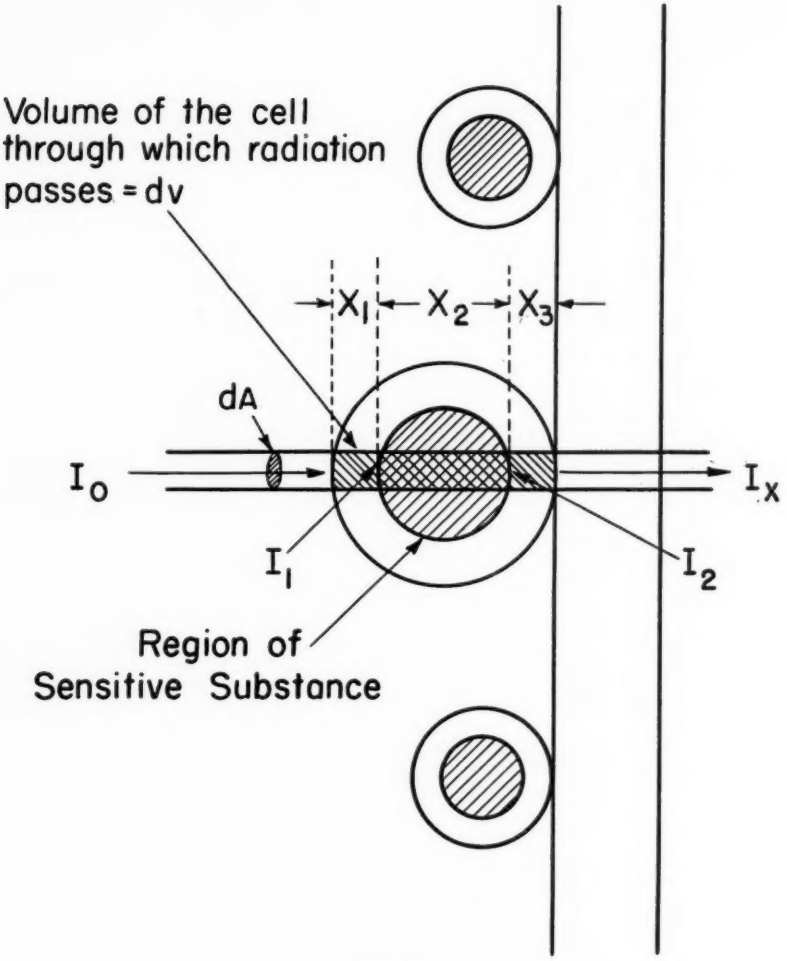

FIG. 7.
HYPOTHETICAL MODEL ILLUSTRATING THE IRRADIATION OF MICROORGANISMS SPREAD ON THE SURFACE OF A MEDIUM.

The absorption coefficients, a_1, a_2, a_3, etc., represent, in each instance, the product of the specific absorption per unit concentration and the concentration.* The two brackets on the right hand side of equation (7) represent transmission factors corresponding to attenuation of intensity as a result of (1) scattering and reflection and (2) absorption in inactive substances. If we call the lumped transmission factors respectively A and B, equation (7) may be written as follows:

$$I_1 = I_0AB \tag{8}$$

If the inactive substances are distributed non-uniformly throughout elements of the path length x_1, (as would be expected in general) the analysis of the problem is more complicated, but the final result is the same; we shall therefore consider only the simpler case.

c. Expression for Absorption by the Active Substance—Let x_2 be the path length through the essential substance and let its absorption coefficient be β. Then if I_2 is the intensity of radiation as it leaves the essential substance.

$$I_2 = I_1e^{-\beta x_2} \tag{9}$$

and the amount of energy, E_a, absorbed in the essential substance is

$$E_a = (I_1 - I_2)tdA = I_1\,(I - e^{-\beta x_2})tdA \tag{10}$$

where t is the time of exposure in seconds. But from equation (5)

$$n = \gamma\frac{E_a\lambda}{hc} = \frac{\gamma\lambda}{hc}\,I_1\,(1 - e^{-\beta x_2})tdA \tag{11}$$

And for a standard biological effect at two different wavelengths λ and λ',

$$\frac{\gamma'\lambda'}{hc}\,I'_1\,(1 - e^{-\beta' x_2}t'dA = \frac{\gamma''\lambda''}{hc}\,I''_1\,(1 - e^{-\beta'' x_2})t''dA \tag{12}$$

*When monochromatic radiation of initial intensity Io passes through a distance x of a solution of a substance having an absorption coefficient S and a concentration c', the intensity Ix, of the radiation as it leaves the substance is $I_x = I_0e^{-\delta c' x}$. This expression is known as the *Lambert-Beers* law. In equation (7), the coefficients δ and c' are lumped for convenience in a single constant, a, where $a = \delta c'$. American and English workers usually use 10 as the exponential base, thus $I_x = I_0 10^{kc'x}$, where k is known as the extinction coefficient.

In this expression, h, c, and dA, are constants independent of λ, whence

$$\gamma'\lambda'I'_1\,(1-e^{-\beta' x_2})t' = \gamma''\lambda''I''_1\,(1-e^{-\beta'' x_2})t'' \qquad (13)$$

or

$$\frac{I'_1 t'}{I''_1 t''} = \frac{\gamma''\lambda''\,(1-e^{-\beta'' x_2})}{\gamma'\lambda'\,(1-e^{-\beta' x_2})} \qquad (14)$$

substituting for I_1 in (13) from (8):

$$\frac{I'_o t'}{I''_o t''} = \frac{A''B''\gamma''\lambda''\,(1-e^{-\beta' x_2})}{A'B'\gamma'\lambda'\,(1-e^{-\beta'' x_2})} \qquad (15)$$

It should be noted that equation (15) involves the assumption that the reciprocity law holds for the photobiological effect over the range of times employed.

d. Simplifying Assumptions—In the applications of action spectra, it is usually assumed that A and B are sufficiently independent of wavelength so that they may be considered to be constants (that is to say, $A' = A''$ and $B' = B''$). This is approximately true even if A and B vary with λ provided the attenuation of intensity as a result of these terms is less than about 10 per cent for any of the wavelengths considered (that is, if $AB = 0.9$ or more for all wavelengths, so that the maximum possible variation of AB as a function of wavelength is from 0.9 to 1.0). It is usually assumed further, that the quantum efficiency, δ is a constant for all wavelengths considered. If these assumptions are valid, equation (15) simplifies to

$$\frac{I'_o t'}{I''_o t''} = \frac{\lambda''}{\lambda'}\,\frac{(1-e^{-\beta'' x_2})}{(1-e^{-\beta' x_2})} = \frac{\lambda''}{\lambda'}\,\frac{(1-10^{-k''cx_2})}{(1-10^{-k'cx_2})} \qquad (16)$$

where k is the extinction coefficient of the essential substance and c is its concentration.

For a small range of wavelengths for which λ' is essentially the same as λ'',

$$\frac{I'_o t'}{I''_o t''} = \frac{(1-e^{-\beta'' x_2})}{(1-e^{-\beta' x_2})} = \frac{(1-10^{-k''cx_2})}{(1-10^{-k'cx_2})} \qquad (17)$$

Under these conditions, the reciprocals of the incident energy densities, I_0t, required to produce the standard biological effect are proportional to the fractions of the radiation, $(1—10^{-kcx_2})$, absorbed by the essential substance. In simplifying equation (16) to yield equation (17), it is assumed that the magnitude of the quantum, $h\nu$, varies very little throughout the wavelength region under consideration. This assumption does not involve great error within a limited range, as for example from 2200 to 2900 A., and does not greatly change the appearance of the action spectrum within such a range, as shown in Fig. 6. Nevertheless, the simplification in computation made possible by this assumption is trivial, and it would seem preferable, therefore, to compute and present action spectra on a relative number of quanta basis rather than on a relative energy basis.

e. Relation of Fractional Absorption to Extinction Coefficients— When the fraction of the radiation absorbed by a particular subtance in a given path length is plotted as a function of wavelength, the shape of the resultant curve depends upon the concentration. This is illustrated in Fig. 8, which shows absorption curves of yeast nucleic acid, plotted on a per cent absorption basis, for various concentrations in a 1 cm. path length. A similar result is obtained, of course, if the path length is varied and the concentration is kept constant, or if the product $c'x$ is varied. Likewise, if the percent absorption relative to maximum absorption is plotted for different concentrations, absorption curves of different shapes result, as shown in Fig. 9. Strictly, therefore, the reciprocals of the incident energies required at different wavelengths to produce a given biological effect should be compared with the relative absorption at different wavelengths of the sensitive substance for the product $c'x$, equivalent to that occurring in the irradiated organism.

In general, there is no accurate way of determining the average effective value of $c'x$ for the sensitive substance in a group of organisms subjected to radiation. Fortunately, however, the relative absorption of a substance at different wavelengths approaches the relative values of its extinction coefficients at those wavelengths as the value of $c'x$

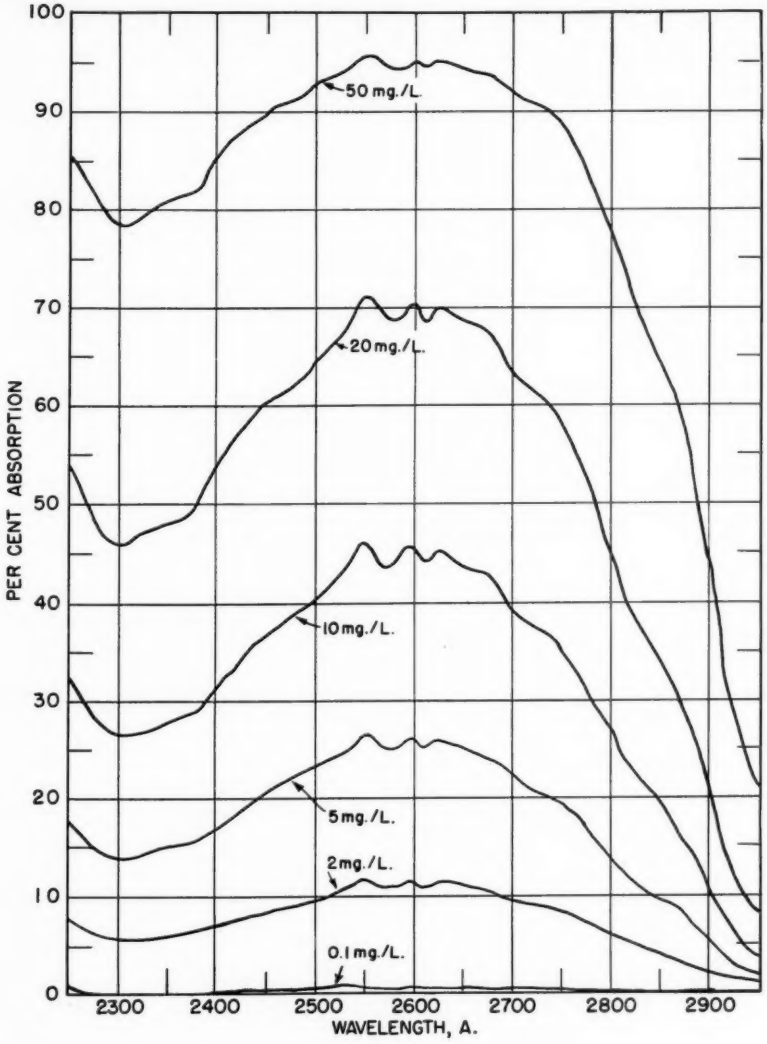

FIG. 8.
PER CENT ABSORPTION OF RIBOSE NUCLEIC ACID AT VARIOUS CONCENTRATIONS FOR 1 CM. PATH LENGTH.

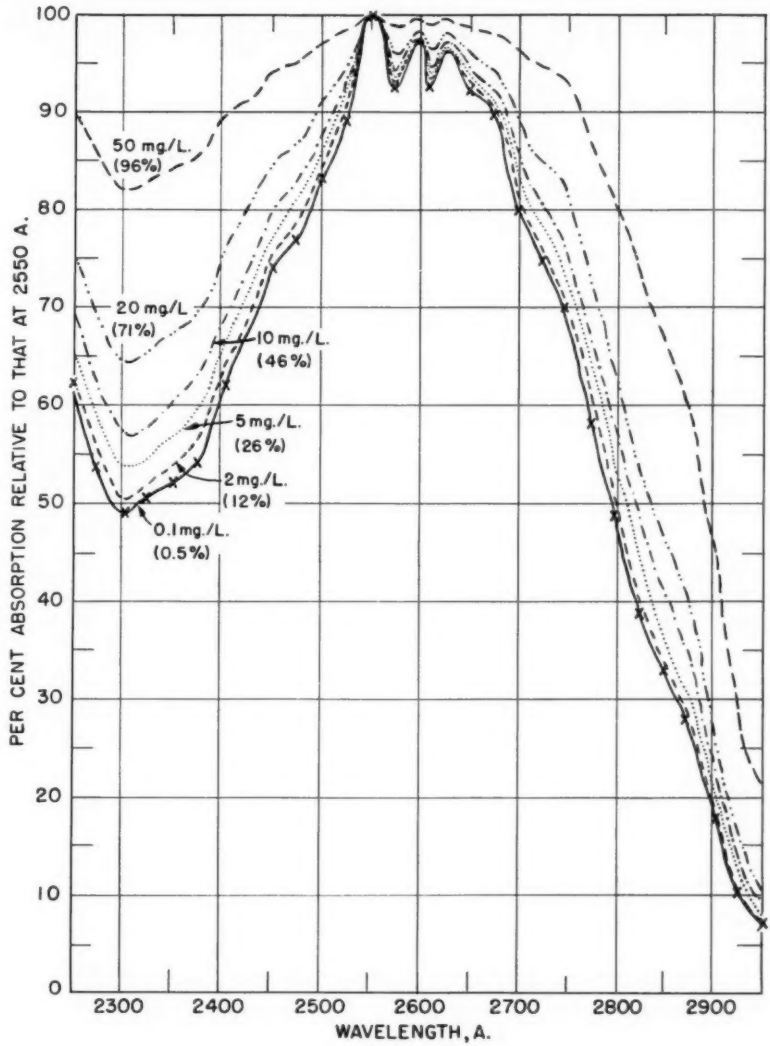

FIG. 9.

ABSORPTION OF RIBOSE NUCLEIC ACID AT VARIOUS CONCENTRATIONS FOR 1 CM. PATH LENGTH, RELATIVE TO THE ABSORPTION AT 2550 A. THE FIGURES IN PARENTHESES REPRESENT THE APPROXIMATE PER CENT ABSORPTION AT THE MAXIMUM (2550A.). THE CROSSES REPRESENT EXTINCTION COEFFICIENTS.

is decreased.* For values of $c'x$ such that the maximum absorption at any wavelength is less than 25 per cent, the maximum deviation of relative absorption values from relative extinction coefficient values is less than 10 per cent, as illustrated in Fig. 9. In experiments such as those of Gates (1929 a, b; 1930) in determining action spectra for the inhibition of division in *E. coli,* the maximum over-all absorption and scattering by all substances in the cell accounts for an intensity attenuation of less than 25 per cent (Gates, 1930), and comparison of the relative reciprocal energies required to produce the standard biological effect with the relative extinction coefficients of substances suspected of being responsible for the effect appears to be justified. In instances of larger cells, in which the attenuation of intensity by absorption in the sensitive substance is quite large, comparison of relative reciprocal energies with relative extinction values may not be warranted.

4. *Conclusions Regarding Action Spectra*

The foregoing analysis applies to situations in which a single layer of cells, spread on a surface, is subjected to radiation. More complex

*Thus $I_a = I_o - I_x$, where I_a is the reduction in intensity as a result of absorption. Also, $\delta c'x = \log_e I_o - \log_e I_x$ for monochromatic radiation if the absorbing substance is in solution. But $-\log_e I_x = -\log_e (I_o - I_a)$, since $I_x = I_o - I_a$ (see above). Expansion of $-\log_e (I_o - I_a)$ by Maclaurin's theorum yields

$$-\log_e (I_o - I_a) = -\log_e I_o + \frac{I_a}{I_o} + \frac{I_a^2}{2I_o^2} + \frac{I_a^3}{3I_o^3} + \ldots + \frac{I_a^n}{nI_o^n} + \ldots$$

whence:

$$\delta c'x = \frac{I_a}{I_o} + \frac{I_a^2}{2I_o^2} + \frac{I_a^3}{3I_o^3} + \ldots + \frac{I_a^n}{nI_o^n} + \ldots +$$

or in terms of extinction coefficients, k, where $k = 0.4343\delta$

$$k = \frac{0.4343}{c'x}\left[\frac{I_a}{I_o} + \frac{I_a^2}{2I_o^2} + \frac{I_a^3}{3I_o^3} + \ldots + \frac{I_a^n}{nI_o^n} + \ldots\right]$$

For two wavelengths λ' and λ'', the corresponding extinction coefficients, k' and k'', therefore have the following ratio (assuming c' and x to be constants independent of wavelength) if conditions are such that all the terms in brackets except the first can be neglected: $k'/k'' = (I'_a/I'_o)/I''_a/I''_o)$. If (I_a/I_o) is small, little error is introduced by neglecting all terms in brackets but the first. Thus when $(I_a/I_o) = 0.1$, the sum of all terms after the first is less than 6% of the first term. Under these circumstances, the ratio of extinction coefficients at different wavelengths approximates closely the ratio of fractional absorptions at these wavelengths.

situations, as for example instances in which suspensions of cells are irradiated, with or without agitation of the suspension, may be analyzed in a similar manner, with analogous results.

The analysis shows that the action spectrum technique involves several assumptions, usually including the following: (1) the standard photobiological effect observed is, on the average, attributable to photochemical change in a given number of molecules of an essential substance per cell, (2) the quantum efficiency of the photochemical process is independent of wavelength within the region explored, (3) the attenuation of intensity of radiation before it reaches the sensitive substance, as a result of scattering, reflections, and absorption by noncritical substances, is either independent of wavelength or is small enough in magnitude so that wavelength dependence does not materially influence the results, (4) the relative absorption of suspected sensitive substances in the cell as a function of wavelength can either be estimated with sufficient accuracy or can be assumed to be equivalent to the relative extinction coefficients of the suspected substances, and (5) the reciprocity law is valid for the range of times employed in the experiments.

In view of these many assumptions, and of further difficulties in the biological aspects of the experiments, it may seem surprising that action spectra have yielded any useful information. Fortunately, if one is fully aware of the perturbing factors, one can hold them within reasonable limits in many instances, so that useful interpretations of the data are possible. For example, action spectra focussed attention very early on the apparent role of purines and pyrimidines in abiotic effects of far ultraviolet radiation and in the production of mutations thereby.

Experience has shown that the technique can yield useful information when judiciously applied. Probably the chief pitfall to be avoided is the assumption, apparently not uncommon, that because the method involves careful measurements with elaborate physical equipment it must necessarily yield meaningful information.

5. Further Possibilities for Investigating Specific Blocking and Accelerating Effects

It seems appropriate to point out some experimental procedures that might lead to more specific data in investigations of direct blocking

or accelerating effects of ultraviolet radiation on biological processes.

First, as stressed earlier, it seems of primary importance to obtain additional quantitative information concerning the photochemistry of important biochemical substances.

Second, it appears that greater attention could be given to eliminating influences of reciprocity-law failure in photobiological investigations. This purpose might be accomplished by employing experimental conditions in which the intensity rather than the time of exposure is varied to obtain different incident energies, now that progress in the development of radiation sources, monochromators, and ultraviolet band-pass filters has made it possible to obtain greater intensities of monochromatic radiation. Lowered temperatures should, theoretically, also reduce the magnitude of reciprocity-law failure.

Third, greater attention might be given to the use of ultraviolet radiation as a blocking agent in the investigation of simpler systems, such as *in vitro* enzyme systems.

Fourth, low-temperature absorption spectroscopy greatly increases the specificity of absorption techniques (see Section VI), and analogous methods might be applied in determining action spectra, at least for simpler systems.

Fifth, ultraviolet microscopy offers the possibility, as yet comparatively unexplored, of irradiating selected portions of cells and tissues for the purpose of investigating the localization of effects. Microirradiation techniques, combined with such special methods of studying cells as phase contrast microscopy and polarized-light microscopy, should yield useful information about the cytological changes induced in organisms by radiation.

V. Effects on Normal Cells of Products from Radiation-Damaged Cells

Ultraviolet radiation is an important agent for studies of the effects of damaged cells on normal cells. In this section, we shall consider various investigations involving such effects.

1. Significance of Effects of Damaged Cells on Normal Cells

a. Use of Ultraviolet to Investigate Repair Processes—The repair processes that ensue after damage to cells or tissues in higher organisms often follow much the same general pattern for a wide variety of

damaging agents. The specific nature of the response appears to be inherent in the nature of the organizational relationships between the cells rather than in limitation of the effect of the damaging agent to a particular biochemical or physiological process in the individual cells. Consequently, a damaging agent that evokes highly specific blocking or accelerating effects in individual cells is not required for investigations of the relationship between damaged and undamaged cells. In fact, use of a comparatively non-specific damaging agent may be desirable; the lack of specificity of far ultraviolet radiation as compared with certain chemical agents that inhibit cellular processes makes the use of ultraviolet favorable experimentally.

b. Variation in Radiation-Damaged Populations—Interest in repair processes following cell damage is, however, only one reason for consideration of the effects of radiation-damaged cells on normal cells. In any cell population of considerable magnitude, it is unlikely that all of the cells will be damaged by radiation at the same time and to the same degree. That this is so was indicated in the discussion of experiments on inhibition of cell division by far ultraviolet radiation, in which it was noted that the percentage of cells inhibited varies markedly with the total radiant energy to which they are subjected. As a specific example, Gates (1929, a) found that the incident energies required at 2660 A. for abiotic action (inhibition of colony formation) on *S. aureus* under the conditions of his experiments varied from 16 ergs per mm.^{2} for minimum observable effects to 350 ergs per mm.^{2} for one hundred per cent killing, a range of approximately twenty to one. When one considers changes induced by radiation in the over-all statistical rate of cell division, respiration, glycolysis, and so on, of a large population of cells, one must therefore take into account the possibility that there may be present comparatively normal, or undamaged, cells that are influenced in their metabolism by the damaged cells.

c. Role of Chemical Factors—Both physical and chemical effects might be involved in giving rise to effects of damaged cells on undamaged cells in a population, but we are concerned here only with chemical factors. In order that chemical substances from damaged cells may influence undamaged cells in an irradiated population, it is essential that the cells be present together in a milieu that will permit diffusion of substances from the damaged to the undamaged cells. If

phenomena of this type exist, one will expect to find evidence of the presence of such substances in the milieu at some stage of experiments. It is therefore quite easy to investigate such phenomena. One has simply to irradiate cells in a liquid suspending medium, separate the medium from the cells, and test the effects of the cell-free medium on populations of undamaged cells in comparison with cell-free media obtained in a similar fashion from control suspensions of undamaged cells.

2. Effects of Suspension Media From Radiation-Damaged Cells

a. Effects on Proliferation and Respiration—In early experiments, Fardon, Norris, Loofbourow and Ruddy (1937) and Fardon, Carroll and Ruddy (1937, a, b) irradiated suspensions of yeast cells in isotonic saline or Ringer's glucose-phosphate solution with far ultraviolet radiation until they no longer respired. When aliquots of these suspensions were added to suspensions of undamaged yeast cells, in growth, oxygen uptake, and fermentation experiments, the rate of proliferation, respiration, and fermentation of the undamaged cells was increased. Cell-free suspension fluids from radiation-damaged cells had several-fold greater stimulating effect than did the damaged cells themselves. Similar results were obtained with x-rays and, in subsequent experiments, with other types of damaging agents (Loofbourow, Cook, Dwyer, and Hart, 1939; Loofbourow and Dwyer, 1938, 1939).

About this time, Harker (1938) reported that irradiation of yeast suspensions with gamma rays leads to the release into the suspending fluid of substances, other than invertase, that increased the rate of sucrose inversion by undamaged yeast cells.

b. Relation of Yield to Physiological Activity—Subsequent investigations, reviewed by Giese (1947) and Nutini (1944), indicated that the activity of the suspension fluids of the radiation-damaged cells is greatest if the cells are subjected to low-level abiotic radiation over a considerable period of time. Similarly, the effect appeared to be enhanced if the suspending medium were favorable to normal metabolism of the cells subjected to radiation damage (Loofbourow and Dwyer, 1940) as shown in Figure 10. These results were interpreted to indicate that at least a part of the activity of the suspension fluids derives from metabolic products of the radiation-damaged cells.

c. Investigations with Bacteria and Tissue Cultures—Somewhat

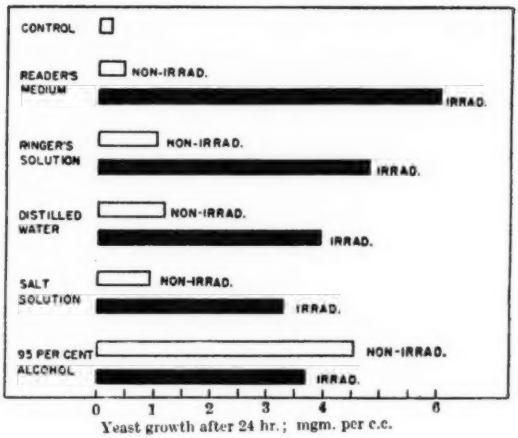

FIG. 10.
EFFECT OF THE SUSPENDING MEDIUM ON THE YIELD OF PROLIFERATION-PROMOTING SUBSTANCES FROM RADIATION-DAMAGED CELLS (LOOFBOURROW AND DWYER, 1940).

similar effects have been found with bacteria (Loofbourow and Morgan, 1940) and with other types of cells. For example, cell-free fluids from radiation-damaged rat-embryo or chicken-embryo tissue were found to increase the rate of division of fibroblasts in tissue cultures (Sperti, Loofbourow and Lane, 1937; Loofbourow, Cueto, and Lane, 1939). In such experiments, however, it was more difficult to separate the effects of cell disintegration products, in both the control and irradiated suspensions, from those attributable to products released by grossly intact, radiation-damaged cells, and the necessity of relying upon area measurements of the tissue cultures as an indication of proliferation rate introduced uncertainties, as will be appreciated by those who are familiar with tissue culture techniques. Typical results of the tissue-culture experiments are shown in Table VII.

d. Investigations with Yeast—The more recent experiments have been carried out with yeast, since it is an easier organism with which to work, especially if the aim is to obtain quantitative results and to investigate the nature of the chemical factors involved. In typical procedures (Loofbourow, Dwyer, and Lane, 1940; Loofbourow, Dwyer and Cronin, 1941; Loofbourow, Webb, Loofbourow, and Abramowitz, 1942), yeast suspensions of *S. cerevisiae*, F. B. strain, in distilled

TABLE VII

GROWTH OF FIBROBLASTS (CARDIAC EXPLANTS FROM CHICK EMBRYO) IN FLUIDS FROM ULTRAVIOLET IRRADIATED AND NON-IRRADIATED CHICK EMBRYO TISSUE
(Loofbourow, Cueto, and Lane, 1939)

	Mean Growth (Area Increase)				
Growth Period hours	In Fluids from Unirradiated Tissue	In Fluids from Irradiated Tissue	Difference of means D	Standard Deviation δ	D/δ
17	0.3	2.2	1.9	0.43	4.4
48	1.7	9.7	8.0	0.66	12.1

water, isotonic saline, or other media are irradiated with full or monochromatic ultraviolet radiation under conditions in which cell death, as indicated by methylene-blue staining or inability to produce colonies, is quite slow, inappreciable mortality occurring in the first three to five hours of irradiation. Control suspensions are kept under similar conditions, except that they are not irradiated. After irradiation, the suspending fluids are freed from the irradiated and control cells by centrifugation, followed by filtration through Seitz or Berkefeld filters. When such preparations are tested for proliferation-promoting effect on yeast cultures, the results are typically as shown in Figure 11. Effects of similar preparations on the endogenous and exogenous respiration of yeast are shown in Figure 12 (Sizer and Loofbourow, unpublished); these effects are blocked by cyanide.

The growth results shown in Figure 11 were obtained with Reader's medium (Reader, 1927), a synthetic medium free from animo acids and vitamins and hence not capable of evoking maximum growth response from yeast. This medium was chosen in order to detect growth-stimulating effects that might be attributable to amino acids and B-factors. However, even when the most favorable of growth media are used, well supplemented with all the growth factors known to be required by yeast, an increased rate of cell division is still observed upon addition of suspension fluids from radiation-damaged cells (Cook and Cronin, 1941; Loofbourow, 1942).

3. *Chemical Nature of Factors; the Possibility of Synthesis in Damaged Cells*

a. Role of B-Factors, Amino Acids, and Nucleotides—Cell-free suspension media from radiation-damaged yeast cells, prepared as

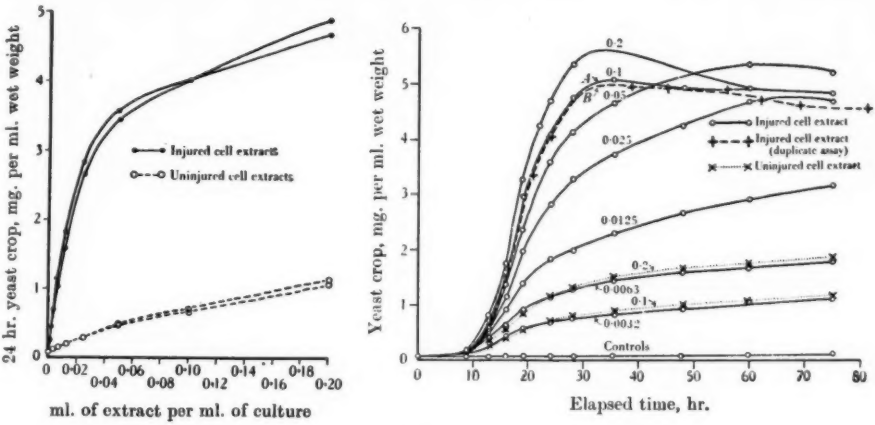

FIG. 11.

TYPICAL EFFECTS OF PRODUCTS FROM RADIATION-DAMAGED YEAST CELLS ON YEAST GROWTH. THE CURVES AT THE LEFT SHOW THE RELATION OF THE 24-HOUR YEAST CROP TO THE AMOUNT OF ADDED MATERIAL. THOSE AT THE RIGHT SHOW THE GROWTH KINETICS FOR VARIOUS AMOUNTS OF ADDED MATERIAL, THE NUMBERS ON THE CURVES REFERRING TO THE ML. OF ADDED MATERIAL PER ML. OF CULTURE (LOOFBOURow, WEBB, LOOFBOURow, AND ABRAMOWITZ, 1942).

described previously, are crystal-clear fluids of light straw color exhibiting marked yellow-green fluorescence in 3650 A. radiation. They are substantially protein-free, as might be expected from the fact that the cells remain grossly intact under the conditions of irradiation (Loofbourow, Cook, and Stimson, 1938; Davidson, 1940). Early evidence indicated that they contain considerable quantities of adenine, guanine, phosphorous, and pentose sugars, suggesting the presence of adenine and guanine nucleotides (Loofbourow, Cook, and Stimson, 1938). This was confirmed by Davidson (1940), who also found a considerable increase in the amino-acid content of the suspension fluids from radiation-damaged cells as compared with those from controls.

Growth experiments with supplemented media (Cook and Cronin, 1941) indicated that the proliferation-promoting effect on yeast of the damaged-cell products is attributable in part to amino acids and vitamin B factors. This has been substantiated by analytical studies (Webb and Loofbourow, 1947; Loofbourow, 1947; Loofbourow, Oppenheim-Errera, Loofbourow, and Yeats, 1947), which show that

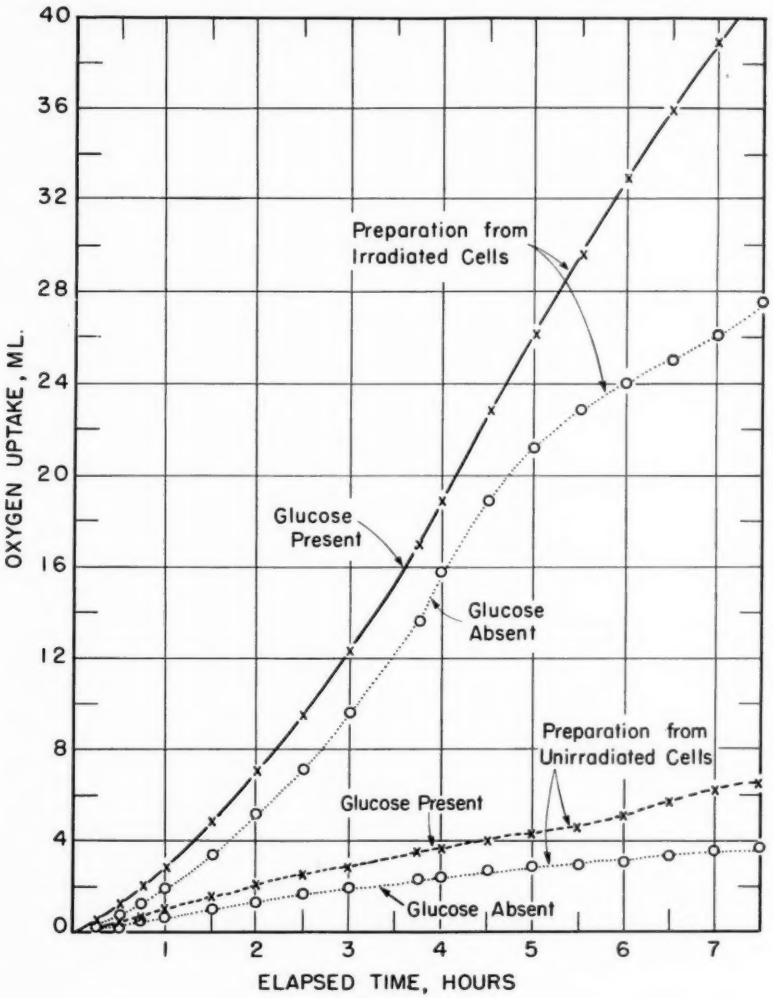

FIG. 12.
EFFECT OF SUSPENSION FLUIDS FROM RADIATION-DAMAGED AND CONTROL CELLS ON THE EXOGENOUS AND ENDOGENOUS RESPIRATION OF YEAST (SIZER AND LOOFBOUROW, UNPUBLISHED).

the changes in the suspension media when yeast is damaged by ultraviolet radiation are quite complex, involving increases in amino acids, B-factors, and nucleotides and nucleosides. All of these factors have been found to contribute to the yeast-growth effect. The growth effect has been duplicated completely by a combination of the factors in the proportions indicated by the analytical data (Table VIII), using

TABLE VIII
ANALYTICAL DATA FOR SUSPENSION FLUIDS FROM RADIATION-DAMAGED YEAST CELLS
(See text for references)

	Content in mg. per ml. of Suspension Fluids from		
	I. Undamaged Cells	II. Radiation-Damaged Cells	Ratio II/I
Nucleotide N	0.45	15.5	34
Nucleoside and free purine N	4.0	21.4	5.4
Amino N	16.7	233.0	14
Protein N	——	1.0	
Biotin	0.001	0.013	13
Folic Acid	0.00028	0.02	71
Inositol	6.0	13.5	2.3
Nicotinic acid	0.7	5.8	8.3
Pantothenic acid	0.045	3.9	87
Pyrodioxin	0.109	0.35	3.2
Riboflavin	0.0077	0.123	16
Thiamin	0.009	0.039	4.3

nucleotide and nucleoside fractions isolated from the preparations as barium or uranium salts and subsequently freed from these metals. The nucleotide and nucleoside fractions are principally adenine compounds, with some guanine also present; their typical spectra are shown in Fig. 13.

b. Possibility of Synthesis—The question naturally arises as to the mechanism that accounts for the presence of proliferation-promoting substances in suspension fluids from radiation-damaged yeast cells. There appear to be at least three possibilities: (1) that the active factors are simply degradation products of substances already present in the cells and are released as a result of increased cell membrane permeability, (2) that the altered metabolism of the radiation-damaged cells leads either to the synthesis of new products or to the accumulation of products that would normally be converted to other substances at a higher rate in the cell, and that these are released into the suspension medium, or (3) that both of these processes occur

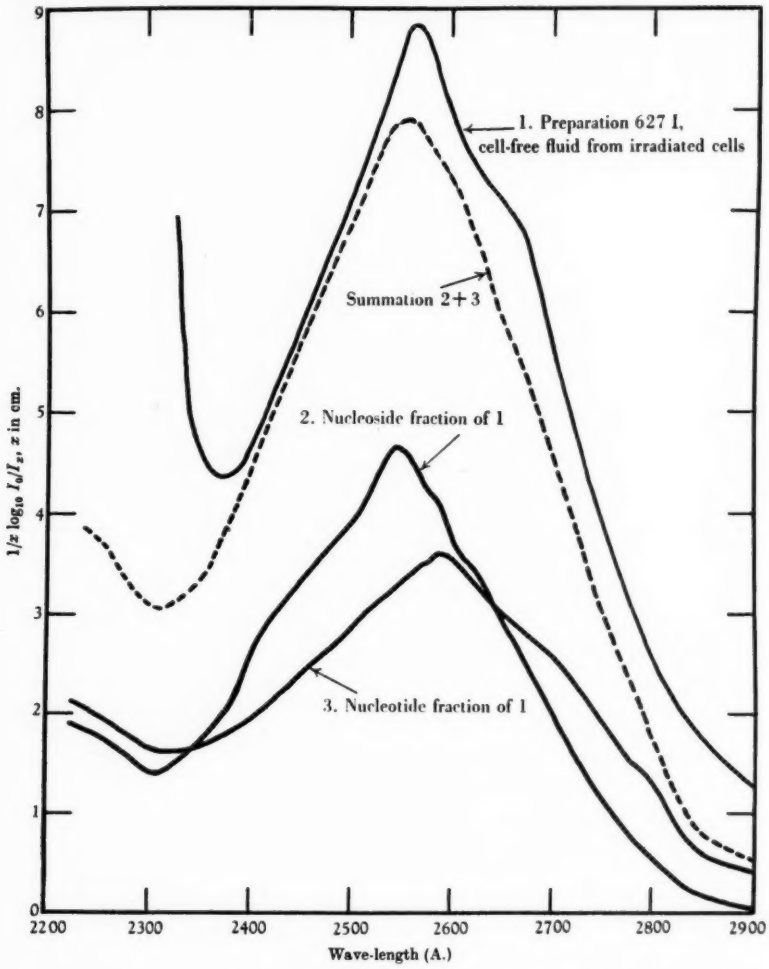

FIG. 13

ULTRAVIOLET ABSORPTION SPECTRUM OF THE CELL-FREE FLUID FROM IRRADIATED CELLS (PREP. 627 I) IN COMPARISON WITH THE SPECTRA OF THE NUCLEOTIDE AND NUCLEOSIDE FRACTIONS THEREOF OBTAINED BY THE KERR & BLISH (1932), KERR (1940) PROCEDURE. THE ABSORPTION COEFFICIENTS, (1/x) LOG (I_0/I_x), OF THE TWO FRACTIONS ARE SHOWN FOR CONCENTRATIONS IN WHICH EACH ML. OF THE NUCLEOTIDE OR NUCLEOSIDE SOLUTION CORRESPONDS TO 1 ML. OF THE CELL-FREE PREPARATION (627 I) FROM WHICH IT WAS PREPARED. (LOOFBOUROW, OPPENHEIM-ERRERA, LOOFBOUROW, AND YEATS, 1947).

simultaneously. That yeast cells become more permeable to P^{32} when irradiated with far ultraviolet radiation has been shown by Hevesy and Zerahu (1946) and permeability phenomena may very likely play some part in the mechanism (compare Loofbourow, 1942 a).

Because of various lines of evidence indicating that synthetic processes are involved in the over-all phenomenon, attempts have been made to determine analytically the amounts of B factors, amino acids, nucleotides and nucleosides in both suspension media and cell residues from irradiated and non-irradiated cell suspensions. When the total residues (including cells and suspension media) were assayed for amino acid content, no appreciable difference resulted from damaged and undamaged cells (Loofbourow, 1947). In the case of B factors, indication of increased over-all yield from radiation-damaged cells was found (Webb and Loofbourow, 1947), but these results might also be attributed to more effective extraction of B-factors from the radiation-damaged cell residues. In the case of nucleotides and nucleosides, the evidence indicates an actual increased over-all yield from the combined cell residues and suspension media of radiation-damaged cells as compared with undamaged cells. (Loofbourow, Oppenheim-Errera, Loofbourow, and Yeats, 1947)

Some other lines of evidence are of interest with respect to the mechanisms involved in this phenomenon. Ultraviolet photomicrographs show increased absorption at 2600 A. in the cytoplasm of yeast cells damaged by far-ultraviolet radiation (Loofbourow and Joyce, 1941). Mitchell (1942) has observed an increase of ribose nucleic acid (but not of desoxyribose nucleic acid) in the cytoplasm of cells exposed to x-rays and gamma rays. Greater yields are obtained in chemical preparations of ribose nucleic acid from ultraviolet-irradiated yeast than from non-irradiated yeast (Loofbourow, Webb, Loofbourow, and Lisco, 1942). Finally, Hevesy (1945) has reviewed work with radioactive phosphorus by himself and others that shows a marked decrease in the desoxyribose nucleic acid content of cells damaged by ionizing radiation. In view of Caspersson's hypothesis that the desoxyribose nucleic acid of the nucleus arises from ribose nucleic acid in the cytoplasm of cells, Hevesy suggests that his results may be attributable to a blocking by ionizing radiation of the processes involved in the transformation of ribose nucleic acid to desoxyribose nucleic acid. This theory explains the inhibition of cell division by ionizing

radiation on the basis of an abnormally low turnover of ribose nucleic acid into desoxyribose nucleic acid, thus preventing the attainment of a sufficient level of the latter in the nuclei of radiation-damaged cells to permit cell division to take place. It is possible that the effects described above with respect to cells damaged by ultraviolet radiation can be explained on an analogous basis. However, abnormal accumulation of ribose nucleic acid in the ultraviolet-damaged cells, accompanied by release of degradation products thereof into the suspending medium, seems inconsistent with the fact that only adenine nucleotides and nucleosides have been found in quantity in the suspension fluids from the ultraviolet-damaged cells.

4. Relation to the Problems of Tissue Damage and Repair

a. Status of "Humoral" Theories—The problem of the relation of normal cells to damaged cells in a large cell population is, of course, of importance in the elucidation of such phenomena as the repair processes that take place following damage to tissues in higher organisms, and the majority of investigators have approached what one may term the "damaged cell problem" from this point of view. The mechanisms of inflammatory processes in animals and of wound healing in plants and animals have received a great deal of attention, and numerous chemical or "humoral" theories of such mechanisms have been proposed. In many instances, such theories have been based upon what seemed to be reasonable experimental evidence at the time, and yet, as Davidson (1945) has pointed out in a recent review of wound healing, none of the "humoral" theories advanced to account for the processes observed in animal organisms has as yet found general acceptance. This situation may be attributable in part to attempts to oversimplify an exceedingly complex series of processes in terms of explanations involving one, or at most a few, chemical entities. Again, little of the work has progressed to the point of actual isolation and chemical identification of the factors presumably responsible for such processes, and until this has been accomplished, it will be difficult to evaluate the validity of the various claims that have been made.

Among the earliest evidence that chemical factors may be responsible for the initial stimulus to tissue repair following cell damage is that of Wiesner (1892), later confirmed by Haberlandt (1921 a, b, c)

among others. Wiesner found that the application of macerated plant tissue to wounds in plants accelerated the repair process. He gave the name "wound hormones" to the chemical factors supposed to be responsible for this effect. English and Bonner (1937) isolated and identified a growth-stimulating substance from beans which they called a "wound hormone", but they were unable to demonstrate that this substance is particularly associated with damaged tissue or concerned with repair processes. As a result of tissue culture investigations, Carrel (1923, 1924) attributed the stimulation of inflammatory processes and tissue repair in higher animals to secretions by leucocytes that he termed "trephones" and that were presumed to be polypeptides and amino acids. Other workers have concluded that the initial stimulus to tissue repair is concerned with sulphydryl compounds (Hammett, 1934), nucleoproteins (Fischer, 1939), or certain nucleic acid derivatives (Calkins, Bullock, and Rohdenberg, 1912).

b. Possibilities for Further Exploration—The investigations of ultraviolet-damaged cells previously described were primarily concerned with effects of radiation-damaged cells upon normal cells, under rather special conditions, and it will be obvious that the results do not necessarily have direct bearing upon such complex problems as tissue repair in higher organisms, overgrowth, and neoplasia. Techniques involving radiation damage of unicellular organisms offer, however, interesting possibilities for further exploration of problems of repair and overgrowth following cell damage. Thus, in the case of the complex tissues of higher plants and animals, it is difficult to separate the damaged portion of the system from the undamaged portion and to isolate with certainty such chemical factors as may mediate between the two. In the case of populations of unicellular organisms damaged by radiation, the situation is considerably simpler. In such instances, it is comparatively easy to damage one population under controlled conditions and then to study the effect either (1) of adding a portion of the damaged population or its milieu to a similar population of undamaged cells or (2) of adding chemical factors isolated from the damaged population or its milieu to an undamaged population.

VI. Effects of Cells on Ultraviolet Radiation

As stated in the introduction, the effects of ultraviolet radiation on cells are always accompanied by effects of the cells on the radiation.

Two effects of the latter type are of especial use in the study of cytochemical problems: conversion of radiation to fluorescence by cellular constituents and changes in the spectral intensity distribution of radiation as the result of selective absorption by chemical substances in the cell. The former is the basis of fluorescence microscopy; the latter of ultraviolet microscopy and microabsorption spectroscopy. In both techniques, of course, it is possible to make use of added materials—that is to say, fluorescent or ultraviolet-absorbing stains—in addition to naturally occurring substances in order to localize structures within the cell.

The techniques and applications of fluorescence microscopy have been described in detail by Haitinger (1937), among others. Caspersson (1936) and his co-workers have established the usefulness of ultraviolet microscopy and microabsorption spectroscopy in the investigation of many problems of cytochemistry and cell physiology. These techniques and their applications have been reviewed, with special emphasis on recent developments, by Scott and Sinsheimer (1948) and Scott (1948). We shall not endeavor to cover the ground so thoroughly reviewed elsewhere; rather, we shall confine these remarks to brief mention of a few current advances in the technique of ultraviolet microscopy and microabsorption spectroscopy that are intended to improve the general usefulness of these methods and to increase the precision and significance of the data obtained.

1. *Achromatic Ultraviolet Microscopes*

Achromatic ultraviolet microscope optics are under development in several laboratories (Lee, 1947; Burch, 1945 a, b, 1946, 1947; Loofbourow, 1948; D. Grey, the Polaroid Corporation, unpublished). These optics are based on the use of reflecting elements alone, or in combination with refracting elements, for the objectives, condensers, or both, of ultraviolet microscopes. Several objectives have been designed and constructed along these lines that are entirely free from chromatic aberrations or sufficiently so to permit one to focus and explore the field of view with visible radiation and subsequently obtain photomicrographs or microabsorption spectra in the ultraviolet region without refocussing. In addition to greatly simplifying the technique and making it easier to apply in practice, these optics have the special advantage of permitting one to avoid radiation damage to cells and

photodecomposition of cellular constituents during the preliminary period of searching and exploring fields.

Figure 14 shows one example of such a microscope system, de-

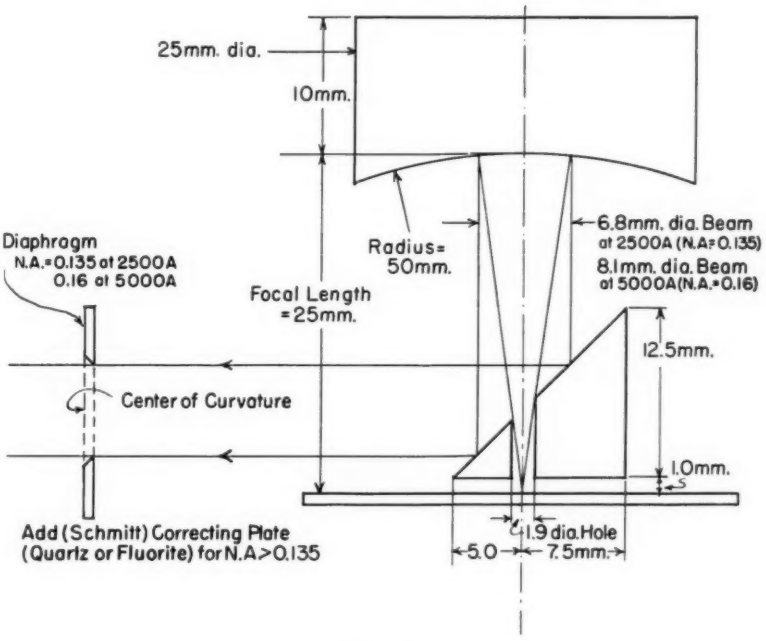

FIG. 14.
ACHROMATIC ULTRAVIOLET MICROSCOPE OBJECTIVE OF SIMPLE DESIGN. NUMERICAL APERTURES UP TO 0.5 ARE FEASIBLE WITH SUITABLE MODIFICATION OF THIS SYSTEM (T. DUNHAM, JR., UNPUBLISHED)

veloped by Dunham (unpublished). It is of the very simplest type, similar to designs of reflection microscopes by Bouwers (1946). Although the resolving power is limited (maximum N.A. with corrector plates about 0.5), this objective is useful for many purposes, and has the advantages of simplicity and inexpensiveness. Burch (1947) has designed and constructed successful reflection objectives of N.A. as high as 1.0.

Many difficult optical problems are involved in these designs. One,

not explored in detail until recently, is the effect on the image of the necessary obscuration of a part of the light beam by condensers and objectives of the reflection type. Without data regarding such effects, the designer is limited in his choice of parameters to those that will result in the lowest possible obscuring ratios. A careful study recently completed by Dunham (1948) shows that linear obscuring ratios up to 0.3 or 0.4 have little effect on images of typical biological specimens; these data will permit greater latitude in future designs.

2. *Polarized and Phase Contrast Ultraviolet Microscopy*

Along with achromatization has gone improvement in optics to permit polarized light microscopy to be carried out comparatively easily in the ultraviolet. The dichroism of many cellular constituents in the ultraviolet, and the increased indices of refraction and more marked birefringence in other substances, make this technique of special interest. Figure 15 is a polarized light photomicrograph showing dichroism of dimethyl thymine crystals in the ultraviolet.

The increased indices of refraction and increased dispersion toward the ultraviolet suggest the usefulness of phase contrast microscopy for certain special purposes in this region of the spectrum. Burch (personal communication) has undertaken a study of this possibility, and similar investigations are under way in our laboratories.

3. *Developments in Microabsorption Spectroscopy*

One of the difficulties of microabsorption spectroscopy in the ultraviolet in the past has been limitation of the measurements to a few wavelengths corresponding to strong mercury arc or spark lines. The use of achromatic objectives (permitting wider spectral bandwidths to be employed), improved monochromators, and more intense sources of radiation, together with newly designed electronic radiation-measuring devices of great sensitivity, stability, and freedom from "noise", permit the continuous determination of microabsorption spectra at all wavelengths throughout the ultraviolet region (Sinsheimer, 1948). These improved methods are intended to provide data that will permit one to distinguish between compounds of closely similar absorption in small portions of cells or tissues, in micro quantities of biochemical preparations, or even in single small crystals.

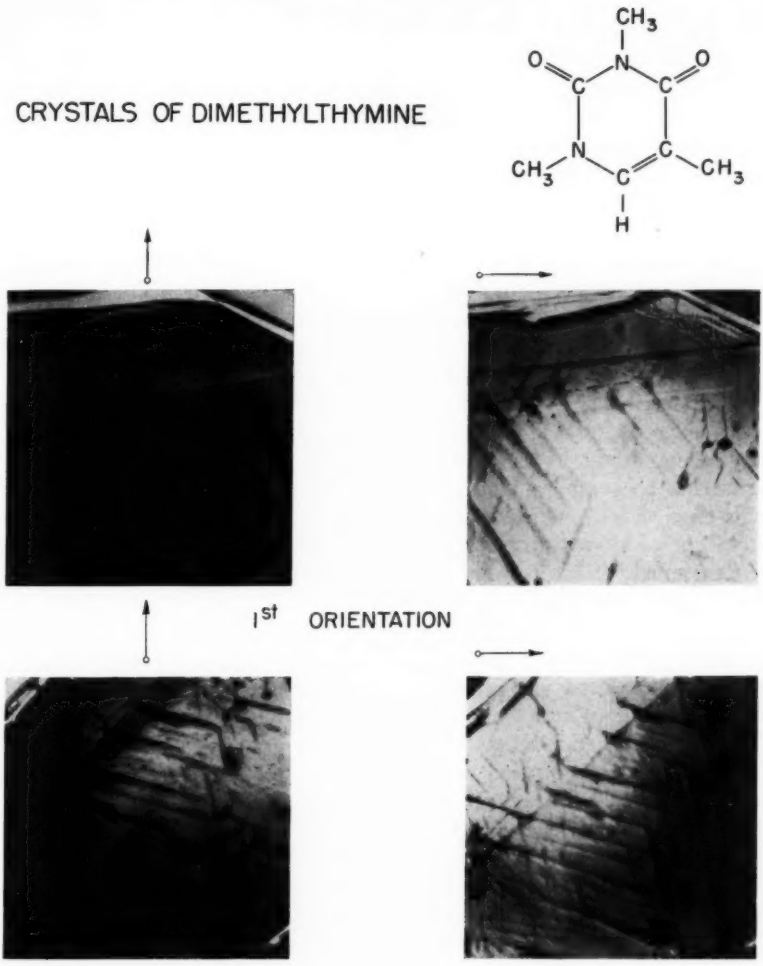

FIG. 15
POLARIZED ULTRAVIOLET PHOTOMICROGRAPHS SHOWING DICHROISM OF DIMETHYL THYMINE CRYSTALS (J. F. SCOTT, UNPUBLISHED).

4. Low-Temperature Spectroscopy

Even when the absorption curve of a substance such as thymine is measured continuously at all wavelengths, the general characteristics of the spectrum at room temperature are so similar to the spectra of other pyrimidines and even of nucleic acids that positive identification is often difficult, if not impossible. From physical theory, one would expect that the spectra of many biologically important molecules should show increased fine structure at the temperatures of liquid nitrogen, liquid hydrogen, or liquid helium, as a result of such factors as reduction in frequency of thermal collisions and decreased randomness of overlapping of the fields of atoms and molecules. That such increased spectroscopic structure is observable at low temperatures, has been demonstrated for pyrimidines and related substances (Scott, Sinsheimer, and Loofbourow, 1948; Sinsheimer, Scott, and Loofbourow, 1948). Figure 16 shows the absorption spectrum of thymine at liquid hydrogen temperature in comparison with the spectrum of the same compound at room temperature. Experiments of this type have thus far been carried out only on the macro scale, but it will be of interest to extend them to microabsorption spectroscopy if the extremely difficult technical problems involved can be surmounted.

5. Color Translation

Color-translation methods (unpublished) are being developed by a group under Land and Blout, at the Polaroid Corporation, whereby the absorption of areas of tissues and cells at several wavelengths may be made visible simultaneously by the use of gradations of visible color to indicate the absorption at each wavelength. Such methods provide a kind of three-dimensional presentation whereby the morphological pattern has imposed upon it a measure of the absorption by different wavelengths at each position in the pattern. A somewhat simplified presentation may be accomplished by taking photomicrographs at different wavelengths in the ultraviolet and then superimposing these as prints in different colors by the wash-off relief process or other color-photography processes. For example, color prints have been made (Scott, unpublished) in which blue corresponds to absorption at 2600 A. and red to absorption at 2800 A., these being the absorption maxima respectively of nucleic acids and proteins. The results simulate roughly the morphological pattern that would be obtained by the

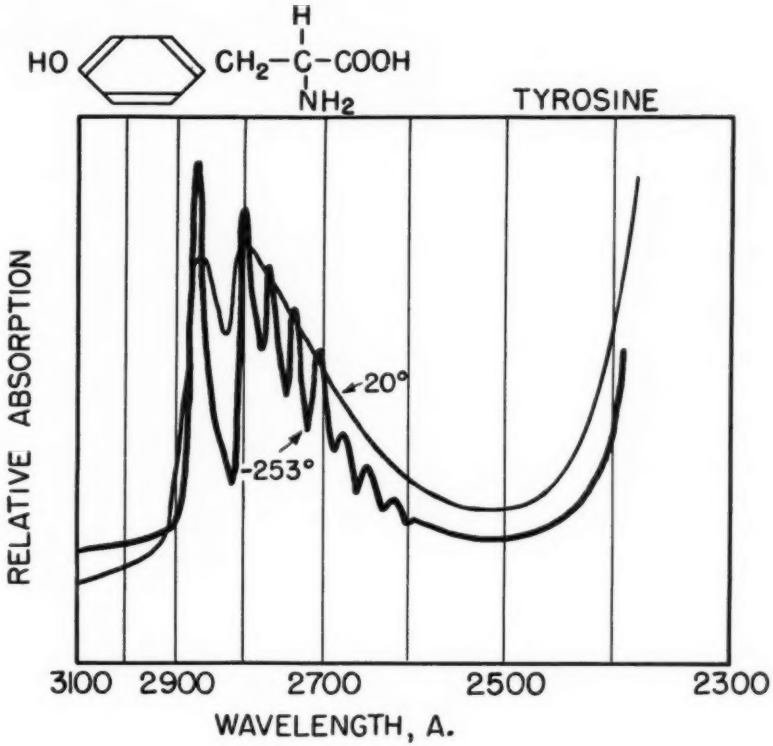

FIG. 16.
ABSORPTION SPECTRUM OF TYROSINE AT LIQUID HYROGEN TEMPERATURE (-253°C.) AND AT ROOM TEMPERATURE (20°C.). (SINSHEIMER, SCOTT, AND LOOFBOUROW, UNPUBLISHED).

use of haemotoxylin and eosin stains, but the possibility that the tissues may be modified in fixing and staining is avoided.

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THE GROWTH IN SURFACE OF THE PLANT CELL WALL

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The rapid elongation of plant shoots in spring is one of the most remarkable phenomena of plant growth. It is known that during this unusual growth (Table I) the meristematic cells extend to many times their original length (Table II), whilst dividing cells are rare in elongating tissues.

TABLE I.
ELONGATION GROWTH OF PLANT ORGANS

Organ	Author	Time of elongation	Velocity of elongation mm/min	cm/day
Stalk of sporogonium of Pellia epiphylla	OVERBECK 1934	3 days		1,0
Root tip of Vicia Faba	SACHS 1873	3 days		1,7
Coleoptile of Avena sativa	WENT & THIMANN 1937	2 days	0,017	(2,4)
Shoots of Bambusa spec.	KRAUS 1895	——	0,4	(57)
Filaments of Secale cereale	SCHOCH-BODMER 1937	10 min	2,5	
Fructification of Dictyophora phalloidea	JOST 1913	15 min	5	

TABLE II.
ELONGATION GROWTH OF PLANT CELLS

Type of cell	Author	Meristematic cell length in μ	Elongated cell length in μ	Elongation	Time of elongation	Velocity of elongation μ/hr
Hypanthium cells of Oenothera acaulis	WEINLAND 1941	8	160	20 x	7 days	0,1
Epidermis cells of wheat root	BURSTRÖM 1942	ca 20	400	20 x	——	——
Coleoptile cells of Zea Mays	BLANK & FREY-WYSSLING 1941	ca 11	156	14 x	4 days	1,5
Coleoptile cells (epiderm) of Avena sativa	AVERY & BURKHOLDER 1936	ca 13	2000	150 x	4 days	21
Seed hairs of Gossypium hirsutum	ANDERSON & KERR 1938	ca 28	28000	1000 x	15 days	78
Filament cells of Anthoxanthum odoratum	SCHOCH-BODMER 1939	79	514	65 x	1 hr	435

This extension growth is provoked by auxins. But I shall not discuss them, because there are so many important problems that must be solved, before the manner of how and where the auxins intervene with the metabolism or with the ultrastructure of the cell can be explained satisfactorily.

1. Cytology.

First of all there is an incredible cytological revolution in an elongating cell. Its length increases 10 to 100 times, and the cytoplasm which originally filled the whole meristematic cell, becomes reduced to a thin layer by the formation of an enormous central vacuole (Fig. 1); the nucleus is pressed against the cell wall; plastids develop from tiny mitochondria like bodies to easily visible leuco-, chromo- or chloroplasts; and the cell wall undergoes an enormous *growth in surface.*

In the classical textbooks the revolutionary character of these transformations has not been recognized. On the contrary, the extension growth has been described as a simple osmotic phenomenon of water attraction by the central vacuole and of wall stretching by the turgor pressure. It is true that there is no elongation growth of cells without turgor, yet this is no proof that the extension is a passive stretch of the cell wall. It is only evidence that a sufficient water supply is necessary for normal growth.

If the extension growth were only a phenomenon of stretching by the formation of the osmotically active central vacuole, the mass of cytoplasm and cell wall substances would have to remain approximatively constant during cell elongation. It is a biochemical task to test whether this is true.

2. Biochemistry.

The difficulty of such an investigation is the choice of a suitable reference value for the analyses. If the results are referred to the dry weight of the tissue—as is generally done—it is impossible to know what is the absolute change of the different constituents of a growing tissue as the reference value is not constant but increases during growth. If then the mass of a constituent of the tissue does not change during growth, the analysis referred to dry weight would indicate a relative diminution, as other constituents increase. Therefore the

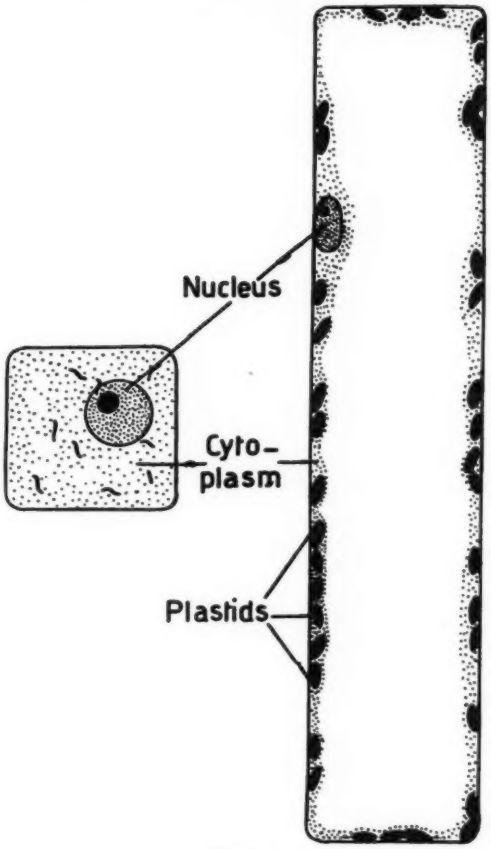

FIGURE 1
CYTOLOGY OF AN ELONGATING CELL.

whole plant, definite organs or single cells, must be chosen as reference value, if we wish to determine the absolute increase of the different cell constituents during growth.

This is evident from Table III which gives the amount of the chemical constituents in the corn coleoptile of different length (Wirth, 1946) referred to dry weight, compared with the results of other

TABLE III.
CHEMICAL COMPOSITION OF CORN COLEOPTILES REFERRED TO DRY WEIGHT

Wall substances	Length of coleoptiles in mm 9	32	55	THIMANN & BONNER	NAKAMURA & HESS
	%	%	%	%	%
soluble in ether	1,2	10,4	7,8	—	2,43
soluble in water (sugars)	44,1	39,5	46,0	—	61,34
hemicelluloses	9,0	14,5	11,0	11,0	—
cellulose	8,3	13,2	13,0	11,9	10,12
pectin	2,2	4,0	4,7	2,3	—
protein	22,17	15,2	13,1	—	—
ash	6,90	4,4	3,6	—	—
dry weight	100,0	100,0	100,0	100,0	100,0

authors. From this table it would seem, that in the growing cell wall none of its constituents is increasing systematically. This would suggest that no appreciable amount of membrane substances is formed during growth. But the continued decrease of proteins and ash is a warning against such a conclusion. These constituents become "diluted" by the formation of other cell constituents, as is seen from Table IV.

TABLE IV.
CHEMICAL COMPOSITION OF CORN COLEOPTILE IN MG PER COLEOPTILE

Wall substances	Length of coleoptiles in mm 9	32	55
fat and wax (ether extraction)	0,040	0,701	0,975
sugars soluble in water	1,016	2,651	5,704
hemicelluloses (20% H_2SO_4)	0,231	0,973	1,371
cellulose (72% H_2SO_4)	0,191	0,930	1,616
pectin (saponified from CH_3OH)	0,052	0,272	0,580
protein	0,510	1,018	1,631
ash	0,160	0,300	0,444
total	2,200	6,845	12,231
dry weight	2,345	6,755	12,400

In Table IV the results of the analysis are referred to a single coleoptile (Wirth 1946). From these figures it is seen that all the substances of the cell wall: cellulose, hemicelluloses and pectins increase considerably during the growth of the coleoptile from 9 to 32 mm length.

In this way it is demonstrated that during cell extension there is no passive stretching, but an *active growth* of the cell wall.

Not only the wall substances but also the proteins increase (Table IV). This means that there is a pronounced formation of protoplasm

TABLE V.
INCREASE IN N IN γ PER ORGAN DURING CELL ELONGATION

Object	Meristematic organ length in mm	Elongated organ length in mm	total N	Increase in protein N	soluble N
Coleoptile of Zea Mays	2	65	34→630	30→282	2→346
	26	65	256→630	144→282	129→346
Filaments of Secale cereale	2	13	1.83→2.34	1.05→1.43	0.70→0.71
Hypanthium of Oenothera acaulis	4	85	148→586	74→286	72→297

during the elongation growth. Table V shows the same behavior in the filaments of rye and the hypanthium of Oenothera acaulis (Blank & Frey-Wyssling 1940, 1941, 1943). The elongation of the filaments of grasses is very rapid (Table I), so that it is amazing how quickly proteins, which must be considered as protoplasm, are formed from soluble nitrogen compounds. Even if we admit that this transformation occurs before the explosive lengthening (Schoch-Bodmer 1945), it shows that the elongation of cells is intimately linked with plasm formation.

From our investigations it follows that cell elongation is not a passive stretching, but an active growth characterized by an important synthesis of wall substances and a considerable formation of protoplasm.

3. Energetics.

If we consider cell elongation merely as an osmotic phenomenon, the energy involved, A, in this growth can be calculated (Frey-Wyssling 1948a). It is the product of the acting turgor pressure T and the increase in volume ΔV of the cell.

$$A = \int T.dV$$

Fortunately the turgor pressure is fairly constant during cell extension, as Burström (1942) has shown for elongating epidermal cells of wheat roots. For a cell of 38 μ length extending to 475 μ with a turgor pressure of about 3 atmos (diameter of the cylindrical cell = 16 μ) the energy A involved is found to be ¼ erg.

There is another way to calculate this energy by starting from the mechanical properties of the cell wall. The formula used is

$$A = v\int \sigma.d\epsilon,$$

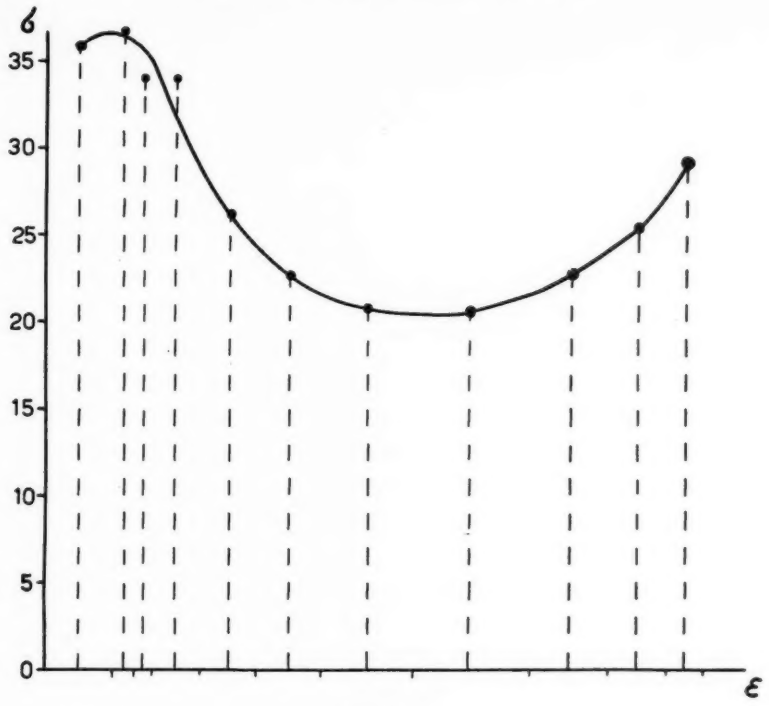

FIGURE 2a.
ENERGY DIAGRAM OF AN ELONGATING EPIDERMAL CELL OF WHEAT ROOT (DATA FROM TAB. VI). σ LONGITUDINAL WALL TENSION IN AT, ϵ SPECIFIC ELONGATION.

where v is the volume of the cell wall involved (not the Volume V of the whole cell), σ is the tension within the cell wall and ϵ is the specific elongation $\Delta l/l$ caused by σ, l is the original cell length and Δl is the increase of length.

The tension σ within the cell wall is by no means identical with the turgor, but it can be calculated from this pressure. In a cylindrical cell it shows a remarkable anisotropy (Castle 1937) for it is twice as big perpendicular to the direction of extension as in that direction which is parallel to the cell axis. The longitudinal tension is $\sigma = rT/2d$, where r means the radius of the cell (8 μ) and d the thickness of the cell wall ($2d = 0.85\ \mu$).

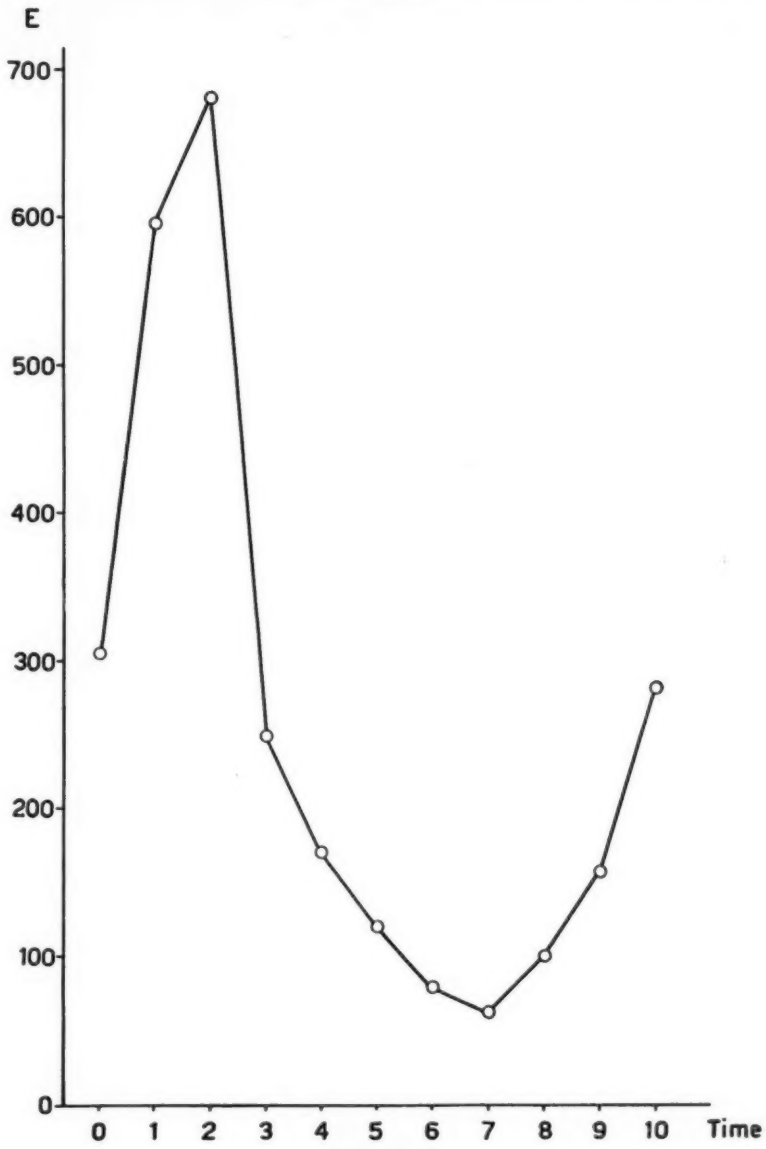

FIGURE 2b.
MODULUS OF ELASTICITY OF THE SAME CELL. E MODULUS IN KG/CM^2 t TIME INTERVALS.

As the turgor pressure does not change fundamentally during cell elongation, it is the same for the tension within the wall. But the specific elongation ϵ is found to vary in a very characteristic manner. During the fast growth of the cell (the so-called "grand period" of Sachs) ϵ becomes ten times as big as at the beginning and end of the cell elongation. The extension growth is therefore primarily not due to an increase in turgor pressure T, which even decreases (Fig. 2a), but to a temporary fundamental change of the elastic properties (Fig. 2b) of the cell wall!

From the specific elongation ϵ and the tension σ the modulus of elasticity E can be calculated ($E = \sigma/\epsilon$). For our example it varies between 600 and 60 kg/cm^2. This value is very low as compared with cellulose fibres where $E = 500000$ kg/cm^2. Thus the meristematic cell wall yields easily to tensions. Even more important than the absolute value of cell wall elasticity is the fact, that this fundamental mechanical property of the cell wall is changed autonomically! In the beginning of the elongation growth it is big, then it decreases suddenly to about 1/10, and finally recovers its initial value towards the end (Table VI). Whether the increase of E in the very beginning of the extension is significant cannot be decided (Fig. 2b).

TABLE VI.

ELASTIC PROPERTIES OF EPIDERMIS CELL WALLS OF WHEAT ROOTS (CALCULATED FROM MEASUREMENTS OF BURSTRÖM 1942)

Time period	I	II	III	IV	V	VI	VII	VIII	IX
	Length of cell		Stretch		Turgor pressure	Tension	Modulus of elasticity	Energy involved	
	plasmolized l in μ	turgescent $l + \Delta l$	Δl μ	$\epsilon = \frac{\Delta l}{l}$	T kg/cm^2	$\sigma = \frac{rT}{2d}$ kg/cm^2	E kg/cm^2	$A = \sigma \cdot \epsilon$ cmkg/cm^3	A/cell cmkg . 10^{-9}
0	34	38	4	0,188	3,8	35,8	304	4,2	3,0
1	48	51	3	0,062	3,9	36,7	594	2,3	2,3
2	60	63	3	0,050	3,6	33,9	679	1,7	2,2
3	73	83	10	0,137	3,6	33,9	248	4,6	7,1
4	100	116	16	0,160	2,9	27,3	170	4,4	9,3
5	122	145	23	0,189	2,4	22,6	120	4,3	11,1
6	144	182	38	0,263	2,2	20,7	79	5,5	16,7
7	168	223	55	0,335	2,2	20,7	62	6,9	24,1
8	221	271	50	0,226	2,4	22,6	100	5,2	24,6
9	295	343	48	0,162	2,7	25,4	157	4,1	25,8
10	430	475	45	0,104	3,1	28,2	281	3,0	27,5
			$\sum_1^{10} = 291$		$T = 3,0$	$\sigma = 28,0$			$\sum_1^{10} = 150,7$

In Table VI, ϵ and σ are given for succeeding periods of growth together with the energy A calculated for each period of elongation. In the beginning A is small and increases steadily because the volume v of the stretched cell wall becomes bigger and bigger. The total of all the partial energies A calculated in Table VI equals 0,15 erg. This value does not differ greatly from the figure 0,25 erg found by our first method. From this we may conclude that the energy involved in the extension of an epidermical cell in the root of wheat is about ¼ erg, as the first method includes a smaller possibility of errors.

To finish our considerations on the energetics of the growth of a single cell, we shall compare the energy A involved with the respiration energy of which this cell disposes. ¼ erg corresponds to about 6.10^{-9} cal. We must compare this value with the content of chemical energy within the sugars available by respiration (the whole osmotic concentration considered as glucose):

	Content of glucose in the cell	Respiration energy available
meristematic cell (length 38 μ)	$1{,}8.10^{-7}$ mg	$6{,}8.10^{-7}$ cal
elongated cell (length 475 μ)	$22{,}8.10^{-7}$ mg	$85{,}3.10^{-7}$ cal
increase during elongation	$21{,}0.10^{-7}$ mg	$78{,}5.10^{-7}$ cal

These figures show, that the energy involved in turgor stretch is only 1% of the respiration energy available in the cell prepared for extension and less than $1^0/_{00}$ of the elongated cell. Therefore it is negligible when compared with the total energy of which the cell disposes. There are probably many processes during growth which consume much more energy. I mention the tenfold increase of the sugar content in the cell during elongation; the transport of this sugar, possibly against a diffusion gradient; the growth of the protoplasm and last but not least the growth in surface of the cell wall. If all this is considered it becomes evident, that the classical conception of elongation growth as a mere turgor stretching, comprises only an unimportant secondary process of the amazing phenomenon of elongation growth.

4. Anaerobiosis.

As so many energy consuming processes are involved in cell elongation, it is interesting to see what occurs if the respiration which furnishes this energy is suppressed. Such experiments have been per-

formed with growing pollen tubes and root hairs of wheat (Kopp 1948). When the oxygen content in the nutritient solutions is lowered beneath a certain level, the growing cells explode. The start of this plasmoptysis can be located. It is due to a break of the cell wall at the very tip of the growing cell (Fig. 3). It is evident that the growth in surface of the cell wall must take place at the same point.

FIGURE 3.
PLASMOPTYSIS OF A ROOT HAIR OF WHEAT (CINEMATOGRAPHS FROM KOPP). THE ARROW ↘ INDICATES WHEN OXYGEN FREE CULTURE SOLUTION IS ADDED TO THE CELL.

This observation can be explained in the following way: there must be two different principles, which control the growth in surface. On the one side a process which softens the cell wall allowing in this way the intercalation of new wall substances, and on the other hand a process which furnishes these substances, incorporates them into the cell wall and hardens the membrane, so that it can stand the turgor pressure. The minimum in the curve of the modulus E shown in Fig. 2b is obviously caused by these two antagonistic processes. The first loosening of the cell wall reduces the modulus of elasticity, whilst the second strengthens the cell wall and readjusts its mechanical properties to the original level. If the maximum of Fig. 2b is reliable, which is not yet proved, it would be caused by the second process preparing the cell wall for extension.

If now we assume that the interruption of respiration inhibits the second process, but not the first, the plasmoptysis by anaerobiosis can be explained.

Criticism might arise against differentiating the growth in surface into two distinct processes by the foregoing arguments. The explosion of root hairs can also be provoked by acids, chlorates or other salts (Lundegårdh 1946, Ekdahl 1948) and not only by anaerobiosis. But those substances interfere probably also with the second process of cell wall extension, because it is a very delicate biochemical phe-

nomenon depending on accurately adjusted pH, redox-potential, enzyme systems (Wanner & Leupold 1947) etc. As compared with these sensitive equilibria, the first process is a phenomenon of hydration and swelling, which is less delicate. For the tip growth (Spitzenwachstum) of cells, such as hyphae, root hairs and pollen tubes, one might consider the first process as superfluous, and consider it only necessary for elongating cells, where it is essential to explain the surprising change of the mechanical properties of the walls. To my mind however elongation growth and tip growth of the cell wall are only two types of essentially the same phenomenon "growth in surface", and therefore I think that the tip growth of any cell is also initiated by the first process.

It is remarkable, that certain protozoa show also plasmoptysis as a response to anaerobiosis. As these organisms have a membrane not of carbohydrates but of protein, I venture the generalization, that in the protoplastic membrane there must be two similar processes of softening and hardening the protein gel during its movement or growth.

5. Submicroscopic morphology.

The behavior of the cell wall whilst its surface is growing must be considered in connection with its submicroscopic morphology. Applying polarized light and the X-ray diffraction method, we have found that the structure of the cell wall, invisible with the ordinary microscope, is a framework of crystalline micellary strands of about 50 Å diameter interwoven by intermicellar spaces. The investigation of plant fibres stained with colloidal silver or gold showed that these micellar strands must form microfibrils (in Germain Grundfibrillen) of about 250 Å diameter (Fig. 4) separated by capillary spaces (Frey-Wyssling 1936, 1937).

The orientation of the submicroscopic elements (micellar strands or microfibrils) can be determined by means of the polarizing microscope. We found that there is a great variety of possible arrangements or textures. All the microfibrils may be arranged parallel (parallel textures) or they may be dispersed or even scattered at random (scattered textures). Cell walls with parallel textures show a very pronounced anisotropy, which appears to be diminished in scattered textures.

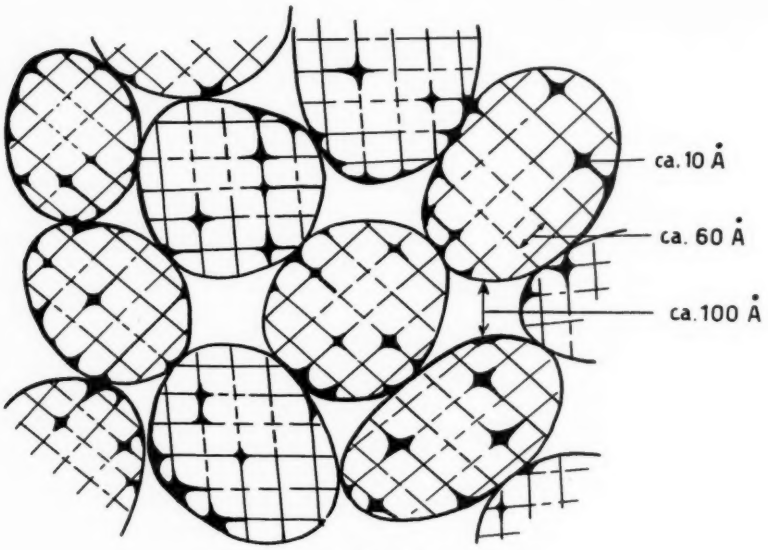

FIGURE 4.
MICROFIBRILS COMPOSED OF MICELLAR STRANDS FROM THE CROSS SECTION OF A BAST FIBRE (FROM FREY-WYSSLING 1937).

Depending on the orientation of the parallel textures in the cell wall, we distinguish fibrillar, circular and screw textures. These textures are restricted to the so-called secondary cell wall which is deposited against the primary wall, when its growth in surface is accomplished. The primary wall has a scattered texture, which is called foliate in isodiametric cells where almost statistical isotropy occurs; in cylindrical cells there is a preference of the transverse direction and this arrangement has been termed *tubular texture* (Frey-Wyssling 1926, 1942, 1948b). A great deal of these textures has been made visible by Bailey (1939) even in the ordinary microscope.

As all the elongating cells, investigated until now, have walls with tubular texture, it is the most important texture for our problem of growth in surface. The increase in length of the cells by growth does not change the preference of the strands for the transverse direction, whereas an artificial stress does (Bonner 1935). To explain this outstanding fact I have advanced the hypothesis that the framework of

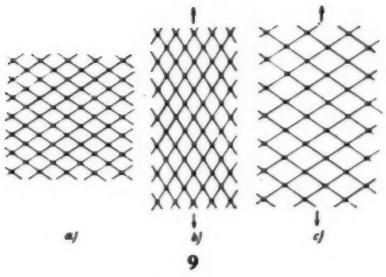

FIGURE 5.
DIAGRAM OF TUBULAR TEXTURE OF PLANT CELL WALLS (FROM FREY-WYSSLING 1936). a) RESTING WALL, b) ARTIFICIALLY EXTENDED WALL, c) GROWTH IN SURFACE

the cell wall must be loosened, its empty spaces enlarged and that new strands must be interwoven (Fig. 5). Of course it is impossible to decide which of the two phenomena, loosening of the framework or neoformation of wall substances, occurs first, because they are interconnected. It is possible that the two manifestations are controlled by different physiological processes as judged from the observations on anaerobical plasmoptysis (Fig. 3).

Today this hypothesis can be proved by means of the electron microscope. Micrographs of shadow casts of primary cell membranes executed by my assistant Dr. Mühlethaler in the Laboratory of Dr. Wyckoff confirm what the indirect methods had predicted about the submicroscopic wall texture: There are microfibrils with a diameter of 200-250 Å, forming a crossed system (Frey-Wyssling 1941, 1948b, Rosin 1946) with the majority lying in the transverse direction. New facts are that these microfibrils are sharply defined and that they seem to be partly interwoven as in a textile fabric (Fig. 6-9). It is remarkable that all the microfibrils electromicrographed until now show a similar diameter, independent of whether they have grown in the primary or the secondary cell wall or even in the extracellular cellulose gel of Bacterium xylinoides (Frey-Wyssling and Mühlethaler 1946). In artificially precipitated cellulose (rayon) no such uniformity occurs. It is therefore a morphogenetic problem how growth stops the further association of cellulose molecules, when the fibrils have reached a definite diameter.

Growth in surface of this system obviously gives rise to new indi-

FIGURE 6.
TUBULAR TEXTURE OF THE PRIMARY WALL OF FLAX FIBRE. (ELECTRON MICROGRAPH BY K. MÜHLETHALER; LAB. OF DR. WYCKOFF.).

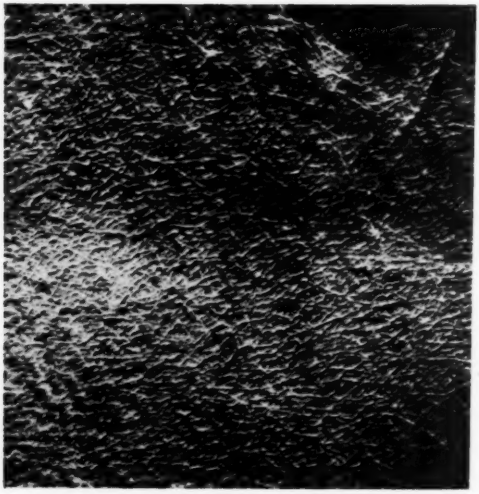

FIGURE 7.
TUBULAR TEXTURE OF THE PRIMARY WALL IN THE CELLS OF A GERMINATING CORN ROOT (ELECTRON MICROGRAPH BY K. MÜHLETHALER; LAB. OF DR. WYCKOFF.).

FIGURE 8.
FIBRILLAR TEXTURE OF THE SECONDARY WALL OF COTTON HAIRS. (ELECTRON MICROGRAPH BY K. MÜHLETHALER; LAB. OF DR. WYCKOFF.)

FIGURE 9.
SIMPLE PIT FROM A CELL IN THE GERMINATING ROOT OF CORN. (ELECTRON MICROGRAPH BY K. MÜHLETHALER; LAB. OF DR. WYCKOFF.)

vidual microfibrils between the existing ones. These must change their distance of separation and for this reason the adhesive forces between the microfibrils must be loosened. This might be attained by a change of hydration, whilst the formation of new microfibrils is a complicated process which involves biosynthesis, respiration and morphogenesis. There is no doubt that the cell wall growing in surface is a living structure and that the theories on elongation growth treating the wall of plant cells as a passive membrane merely stretched by the turgor pressure must be discarded.

When the growth in surface is accomplished and the cell wall becomes consolidated by growth in thickness, its submicroscopical aspect changes fundamentally. In general the wall layers deposited by apposition against the wall which has accomplished its elongation show parallel textures (fibrillar texture in bast fibres, screw textures in cotton hairs and wood fibres, ring texture in certain tracheids etc.). From this statement it might be concluded that a scattered texture is an essential and necessary prerequisite for the growth in surface of membranes. This law will not only hold good for the plant cell wall, but also for the protein membranes of animal cells.

6. Growth in surface within the plant tissue.

The ideal shape of a meristematic cell within a plant tissue (v. Iterson & Meeuse 1941) is a tetrakaidecahedron (a combination of a hexahedron with an octahedron). The wall between adjacent cells consists of three lamellae (Kerr & Bailey 1934), namely the primary walls of the two cells which are cemented together by the middle lamella of intercellular substances (mostly pectins).

It is a problem how this triple laminated wall is growing in surface. Krabbe (1886) assumed, that the middle lamella was plasticized and that every cell had its individual growth. If the increase in surface of adjacent cell facets was unequal they would have to slide one along the other. This *sliding growth* has been criticized by Priestley (1930) because predisposed pits (cp. Fig. 9) would be separated in this way. Also from the viewpoint of the theory that a tissue is acting as an entity, it would seem unlikely that the tissue is disintegrated like a cell colony into its cells which behave differently by assuming individual growth. Therefore it has been supposed that adjacent cell walls usually grow at equal rates, which has been termed *symplastic growth*.

This assumption is supported by the fact that growth in surface of an individual cell facet can vary considerably in its different parts (e.g. epidermal cells of wheat roots, Burström 1942). This recalls the *tip growth* of pollen tubes and root hairs, which is characterized by a restricted local increase in surface at the very end of the cell. Mrs. Schoch-Bodmer (1945) has shown that such local growth occurs likewise, when the top of fibres intrudes between other cells. She called this growth which separates two adjacent cells by splitting the middle lamella of other cells and deposing new primary walls against the separated ones *interposition growth*, which covers the term *intrusive growth* of Sinnot and Bloch.

Microscopical observations have led to the terminology discussed above which comprises at least four different possibilities of the growth in surface of cell walls (elongation growth, tip growth, symplastic growth, interposition growth). The submicroscopic morphology allows us to simplify this complicated system. We have seen that growth in surface is concerned with the intercalation of new microfibrils in an extending framework. This kind of growth has been termed by Nägeli *growth by intussusception* in contrast to the growth by apposition of the secondary cell wall. In my opinion this term includes all kinds of growth in surface, and as it is substantiated today by electron micrographs it can replace all the other terms. I present therefore one of the rare cases where the modern advancement of science suggests a simplification of terminology!

Conclusion

The submicroscopic morphology of cell walls shows that their growth in surface consists

a) in a loosening of an existing framework of microfibrils, and

b) in intercalating new microfibrils into the extending fabric.

Growth in surface of membranes is therefore an essential vital phenomenon intimately linked with the important phenomena of biosynthesis, respiration and morphogenesis.

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HEREDITARY ABNORMALITIES AND THEIR CHEMICALLY-INDUCED PHENOCOPIES*

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For a considerable time our group at Storrs has been concerned with a study of several types of skeletal abnormalities of fowl. In the present review I propose to present first a brief selection of certain aspects of the morphology, inheritance and embryonic development of these traits. This will be followed by a summary of our observations on the experimental production of similar conditions by insulin-treatment of embryos. Finally, various questions of genetics and developmental physiology will be discussed in the light of the recorded facts and with particular reference to the problem of phenocopies, i.e. the simulation of gene effects by the action of extrinsic factors.

RUMPLESSNESS

Two mutations are known in fowl—one dominant, the other recessive—which are responsible for rumplessness, i.e, lack of the tail vertebrae and associated structures, such as muscles, feathers and preen gland (Dunn 1925; Dunn and Landauer 1934, 1936; Landauer 1928, 1945a; Landauer and Dunn 1925). In dominant rumplessness the heterozygous mutant gene appears to find full expression, no morphological differences having been found between hetero- and homozygotes. In the absence of modifiers dominant rumplessness shows clear-cut Mendelian segregation. The expressivity of recessive rumplessness, on the other hand, is very low. The two rumpless mutants are not members of a series of multiple alleles, nor is there any evidence for close linkage.

The morphological expression of both genes for rumplessness is affected by multiple modifiers. In the presence of such modifiers, and depending on their concentration, both forms of rumplessness show a graded series of steps leading to forms which are indistinguishable from the normal phenotype. Modification toward normal tends to be

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more complete among males of the dominant and among females of the recessive rumpless stock.

Among these modified individuals certain morphological features are found which differentiate in an appreciable percentage of cases dominant from recessive rumplessness. For example, caudal lordosis, kypho-scoliosis, lateral compression of fused tail vertebrae and flaring of vertebral margins (in some instances as far anteriorly as the thoracic region) occur among modified recessive rumpless animals.

A more important difference between the two mutants concerns the chemical composition of the adult skeleton. The pelvic bones of recessive rumpless fowl, even of normal overlaps, are deficient in ash and calcium. In comparison with those of dominant rumpless or normal chickens these bones contain an excessive amount of water. Such a hydration exists even in the long bones of the legs, but this is true only of those recessive rumpless animals with phenotypical expression of the tail abnormality; it is not found among the normal overlaps. Pelvis, synsacral vertebrae and uncinate processes of the ribs of late recessive rumpless embryos (with some degree of expression of the mutation) show lagging calcification.

These abnormalities of bone formation presumably account for the frequent occurrence in recessive rumpless stock of kypho-scoliosis. An abnormally high incidence of supernumerary ribs and a reduction of general body size may be expressions of the same fundamental disturbances. No variations of this type occur in the dominant rumpless stock, and, on the whole, one may conclude that the recessive rumpless condition has a more generally systemic effect on the skeleton.

Embryonic development of the two rumpless mutants also shows interesting differences (Zwilling 1942, 1945). In dominant rumplessness formation of the tail structures is prevented by cellular degeneration within the presumptive tail tissue. This cellular degeneration first becomes visible during the second day of development and reaches its climax during the third day. The degenerated cells are extruded, leaving the embryo without the building material for tail formation. These events are of particular interest because they may possibly represent an exaggeration of the normal mode of developmental delimitation of the tail, and also because under the influence of genetic modifiers a quantitative, stepwise control of this cellular degeneration appears to occur in such a fashion that in the presence

of the proper number and the right kind of modifiers degeneration within the presumptive tail tissue is restricted to normal proportions.

In embryos with recessive rumplessness the developmental abnormalities have a later start and take a different course. There is no evidence of increased cellular degeneration in the undifferentiated tail tissue. Instead, abnormalities in tail formation (separation of notochord from nerve cord by fused myotomes and/or somites) become visible at the age of 3½ days or as late as the fifth day. They are succeeded by degeneration and regression of formed tail structures. The later the abnormalities are initiated, the less extreme is the final extent of "rumplessness," but even those recessive rumpless embryos which at hatching or in adult life will become indistinguishable from normal show transitory abnormalities during development.

Modifying genes supply in the case of both rumpless mutants the mechanisms which prevent or repair the damage imposed on the developing embryos by the main genes. The nature of the original disturbance and of the equilibrating effects of the modifiers remains to be determined.

Micromelia and Beak Abnormalities

The group of micromelias holds a curious and interesting place among hereditary abnormalities of fowl (for a more detailed review see Landauer 1948b). At least six different mutations are now known the chief feature of which is a disproportionate shortening of the extremities. In all of them this disproportionate dwarfism is associated with defects of the beak (maxillae) and/or deformities of the skull, and all of them are homozygous lethals.

The Creeper, Cornish and "short upper beak" lethals are semidominant, i.e. they have some heterozygous expression. In heterozygous Creepers the long bones of the extremities are shortened (Landauer and Dunn 1930, Landauer 1931, 1934). This stunting of the long bones is the result of chondrodystrophy-like abnormalities of cartilage formation, different bones being affected to varying degrees. Among homozygous Creeper embryos which survive beyond the stage at which the majority of them succumb, the extremities are in a phocomelic condition, the skull is deformed with orbits which are reduced in size (perhaps the cause of associated eye defects) and with an abnormal beak (Landauer 1933, 1939a). The rate of survival of homo-

zygous Creeper embryos to such late stages is determined by modifying genes (Landauer 1942).

A lethal found among Dark Cornish fowl has in heterozygotes an effect on long bone length similar to, if less extreme than, that of the heterozygous Creeper condition. A severe micromelia characterizes the homozygous embryos which also have a deformed skull and a shortened beak, the upper beak frequently being disproportionately reduced (Landauer 1935, 1939b).

In the third lethal mutation of this group shortening of the upper beak is the most regular feature, but micromelia is a part of the abnormal developmental pattern. The beak defect is completely recessive, but the micromelia appears to have some degree of dominance. By selection and the accumulation of modifying genes it was possible to suppress almost entirely the phenotypic expression and lethal effect of this mutant (Landauer 1941, 1946). In fact, after several generations of selection excessive length of the upper beak was found in a few cases, representing perhaps an instance of genetic overcompensation.

Among the three lethal micromelias of fowl which are completely recessive, one is associated with a shortened upper and a defective lower beak (Asmundson 1942), whereas in the other two, both of which have been described as chondrodystrophy, the embryos have short and curved beaks (Lamoreux 1942, Hays 1944). There is no evidence suggesting genetical relationship between any two of these six micromelia mutations.

Micromelia is not only a condition, however, which, in varying degrees of expression, occurs surprisingly often by independent mutation. It is also produced in chicken embryos by a number of different nutritional deficiencies of the maternal diet, such as inadequacies in manganese (Lyons and Insko 1937), riboflavin (Rozenbakh 1941, Romanoff and Bauernfeind 1942) and biotin (Couch, Cravens, Elvehjem and Halpin 1948). In all these instances a short and curved beak ("parrot beak") is a part of the syndrome. One or the other of these nutritional deficiencies probably accounts for cases of sporadic micromelia. Micromelia of chicken embryos has also been produced experimentally by the destruction of nicotinic acid with injected 3-acetylpyridine and it was possible to reverse this effect by injecting nicotinamide into the yolk sac (Ackermann and Taylor 1948).

POLYDACTYLISM

Polydactylism is another skeletal trait of fowl which is known in several mutant forms. Genetically best known is that polydactylous mutation which furnishes the standard trait of five-toed breeds, such as Dorkings and Houdans. This is a dominant character and is subject to a wide range of modificatory variability. Three other mutations to polydactylism have been found in White Leghorn stocks; they are known as "duplicate" (Warren 1941), "diplopod" (Taylor and Gunns 1947) and "talpid" (Cole 1942). Duplicate is similar in expression to the standard polydactylous mutation, of which it appears to be an allele, but it tends to produce more extreme alterations of the appendicular skeleton. The ranges of expression of the two mutations overlap, but among duplicate fowl polydactylism of the hand is relatively common. Phenotypic expression in the wings of duplicate animals can readily be suppressed by selection. With greater effort selection also accomplishes the reverse, viz., polydactylism of the hands in standard polydactylous mutants. In both stocks, i.e. duplicate and standard polydactylous, the existence of supernumerary fingers is sometimes associated with a partial or complete lack of the radius or with a partial or complete duplication of this bone.

Among the numerous genes which may modify the phenotype of polydactylism there are some which tend to push toe development in one of the following directions: complete suppression of the polydactylous condition, heterodactylism, i.e., the existence of supernumerary toe material on either the left or right but not on the opposite foot, unequal expression of polydactylism on the two feet, and polyphalangism, i.e., a lengthening instead of a duplication of the first toe. Heterodactylism is a common phenotype among heterozygotes but is rare in homozygotes. Complete suppression of the polydactylous condition also occurs much more commonly in heterozygous animals. The incidence of all these forms of expression of polydactylism can be lowered or raised by selection, though the degree of response to selection varies from type to type. Ectrodactylism, i.e., the suppression of normal toes, has a significantly higher incidence in polydactylous than in other stocks, but we know nothing about the genetics of this condition.

Talpid and diplopod, the other two polydactylous mutations, are recessive lethals, affecting both pairs of extremities. In talpid embryos

polydactylism is associated with micromelia, mandible defects and still other abnormalities. In diplopod embryos the first toe is generally replaced by a complement of three toes, constituting a mirror image condition of the normal second, third and fourth toes. The upper beak of diplopod embryos is almost always reduced in size.

Experimental Production of Abnormalities

Rumplessness is one of the most common sporadic abnormalities of chicken embryos. Its incidence varies significantly between different stocks and breeds; in our White Leghorn stock approximately 1.6 percent of the embryos surviving the seventeenth day of incubation are rumpless, whereas in Jungle fowl the frequency was only 0.3 percent (Table 2).

In progenies of genetically normal parents the incidence of rumplessness can be raised appreciably by mechanical shaking of eggs prior to or during the earliest stages of incubation (Landauer and Baumann 1943). In rumplessness of the sporadic type and that produced by shaking we are dealing with an all-or-none effect; intermediate conditions, as they develop under the influence of the modifiers of the two rumpless mutants, do not occur or are extremely rare, even in stocks which are known to carry such modifiers. On the other hand, our data demonstrate that the chances of embryos to become rumpless are influenced by the mother's genotype. This is true for the sporadic cases as well as those produced by shaking. In fact, these two sources of rumplessness appear to have some denominator in common, the eggs of hens with a high sporadic incidence of rumplessness being also particularly susceptible to the effects of shaking ($\chi^2 = 6.09$, d.f. $= 2$). Physical damage to the blastoderm and its after-effects are likely to be the major causes in both instances. It may be concluded that two separate groups of genetic agencies exist: one rendering embryos susceptible to such physical causes of rumplessness as shaking, the other (viz. genetic modifiers) furnishing developmental mechanisms which tend to overcome the effects of the rumpless genes.

Rumplessness may be produced by a great variety of chemicals acting upon the early embryo. In most instances only a small, if significant, fraction of treated embryos responds in this fashion. Certain substances, however, have a rather specific rumplessness-inducing

TABLE 1

THE RESULTS OF EXPERIMENTS REPORTED BY ANCEL AND LALLEMAND

Substance tested	Dose mg.	Number of eggs	Rumpless (without other abnormalities) %	Rumpless with other associated abnormalities %	Intermediate rumpless (without other abnormalities) %	Intermediate rumpless with other associated abnormalities %	Total of rump abnormalities %
Sulfarsphenamine	0.05-0.1	38	0	2.6	5.2	0	7.8
Sulfamyl chrysidine	2	35	0	0	8.5	0	8.5
Trypan blue	0.05-0.1	19	0	0	10.5	0	10.5
Germanin (Bayer 205)	0.05-0.1	28	0	0	17.0	0	17.0
Sodium cacodylate	0.5-2	129	0	13.1	3.8	0	17.0
Sodium methyl arsenite	6-10	49	8.1	12.2	10.2	2	32.5

effect. Ancel and Lallemand (1940-1941, see also Ancel 1945a) have reported the results reproduced in table 1. In their experiments the embryos (breed not given) were treated at the age of 48 hours by direct application of the test solutions. If the small samples are to be trusted, sodium methyl arsenite, which gave by far the highest incidence of rump defects, was the only one of the tested substances that produced at least some cases of complete absence of the tail without inducing at the same time other abnormalities.

We have found that insulin, injected into the yolk sac, leads to a high incidence of rumplessness. The results of such treatment, as far as rumplessness is concerned, vary with the dose of injected hormone, with the time of treatment, and with the genetic constitution of the stock from which the eggs came (Landauer 1945b). Insulin-induced rumplessness may be complete or partial.

The incidence of rumplessness increases with the dose of injected insulin. Between 1/20 of one unit and five units of insulin the dose-response curve is a straight line if incidence of rumplessness, expressed in probits, is plotted against logarithms of dose (Landauer and Bliss 1946). These results suggest that some developmentally important function is being interfered with in a quantitative manner. In White Leghorn stock 2 units of insulin, injected prior to incubation, result in approximately 28 percent of rumplessness among the survivors of the seventeenth day, whereas the corresponding figure for 5 units is about 39 percent. These figures do not show the full effect of insulin since there is some selective mortality of rumpless embryos during early stages of development. The effectiveness of insulin in suppressing tail formation rises from before the start of incubation to a maximum at about 31 hours and approaches zero toward the end of the third day.

Various breeds and stocks differ remarkably in their response to insulin (Table 2), the susceptibility of White Leghorn embryos being three times that of Jungle fowl embryos. It should be noted that the three stocks which, next to Jungle fowl, show the highest resistance to insulin (during the period when rumplessness is induced) have certain peculiarities in common. In one of them selection had occurred against the expression of skeletal abnormalities which originally had been lethal (short upper beak with micromelia); the feature of the second stock (Creeper fowl) is a hereditary micromelia and the pres-

ence of genes modifying this micromelic condition; in the third stock (Buff Orpington) the breeders' standard calls for "rather short lower thighs and shanks" as a breed characteristic. The possible significance of this situation will be discussed later. Variations in susceptibility to insulin exist within as well as between breeds, and it was possible to establish by selection high and low response lines of White Leghorn fowl which differ widely in their incidence of rumplessness following insulin treatment.

In reciprocal crosses between breeds with low and high resistance to insulin-induced rumplessness the F_1 embryos resemble the maternal stock in incidence of rumplessness following insulin treatment. This is shown by the following figures which give the results of the injection of 2 units of insulin prior to incubation in percent of rumplessness among survivors of the seventeenth day.

Leghorn	25.7 ± 3.0
Leghorn ♀ x Jungle ♂	26.7 ± 3.5
Jungle ♀ x Leghorn ♂	9.3 ± 2.3
Jungle	9.3 ± 2.9

In evaluating these data it must be remembered that the eggs of Jungle fowl (fresh weight) are only about 67.6% the size of those of our Leghorn stock, and that, therefore, they received a relatively higher dose of insulin.

There is a possibility that stock differences in the tendency to produce sporadic rumplessness are related to the differences in response to insulin which have been observed in the reciprocal crosses between Leghorn and Jungle fowl. The existence of such stock differences is evident from the data in Table 2, but we have been unable to discover a consistent relationship between the frequency of occurrence of sporadic rumplessness and the incidence of its appearance in response to insulin.

The skeletal features of adult fowl with insulin-induced rumplessness bear a marked resemblance to animals from the recessive rumpless stock. This similarity is not confined to features of the caudal skeleton, but includes the occurrence of synsacro-thoracic scoliosis, flaring margins of certain vertebrae, and the occasional presence of one pair of poorly-formed supernumerary ribs. Chicks which failed to hatch show more extreme skeletal defects, such as lack of calcification in all

TABLE 2

INCIDENCE OF RUMPLESSNESS IN VARIOUS BREEDS AND STOCKS OF FOWL. SPORADIC OCCURRENCE AND FREQUENCY AFTER 2 UNITS OF INSULIN PRIOR TO INCUBATION AMONG SURVIVORS OF SEVENTEENTH DAY OF DEVELOPMENT.

Stock	No treatment	Two units insulin
White Leghorn	1.6 ± 0.4	28.0 ± 1.5
Light Brahma	0[1]	21.2 ± 2.4
Black Jersey Giant	0[2]	17.2 ± 3.0
Creeper	0.2 ± 0.2	14.0 ± 1.6
Homozygotes for "short upper beak" lethal (selected against expression of mutation)	0.8 ± 0.5	10.5 ± 1.6
Buff Orpington	0.3 ± 0.3	10.0 ± 1.8
Jungle	0.3 ± 0.3	9.3 ± 2.9

[1] N = 357
[2] N = 215

vertebrae posterior to the cervical region, the occurrence of as many as nine pairs of ribs or a reduction of ribs below the normal number (Landauer 1947a). A study of the embryology of insulin-induced rumplessness (Moseley 1947) revealed that even more complex abnormalities prevail in early stages of development. About two-thirds of all embryos with caudal abnormalities have some form of ourentery (parts or all of the axial structures of the tail deviating into the hindgut and its derivatives), together with an abortive tail. All of these embryos presumably die during the first two weeks of incubation. The majority of those embryos, however, which would have survived to later stages and a part of which would have hatched were characterized by abnormalities very similar to those of the recessive rumpless stock. It appears, therefore, that this latter group of insulin-induced rumplessness represents phenocopies of the recessive mutant not only in superficial morphological resemblance, but in physiological relatedness.

It has been stated earlier that *micromelia and beak defects* can be produced in chicken embryos by certain dietary conditions. Chemical treatment in early developmental stages may lead to the same result. Ancel (1945b) applied eserine sulfate and certain sulfa drugs to 48-hour embryos and found that after eserine and sulfanilamide (0.5 and 1.25 mg. respectively, per embryo) all survivors of the ninth day showed micromelia, often associated with a short and curved ("parrot") beak. The response to these drugs was more extreme in Sussex

fowl than in Leghorns. One expression of this greater susceptibility was the occurrence of syndactylism in some of the Sussex embryos; another was that among micromelic embryos those of the Leghorn stock had a lower incidence of deformed beaks.

A comparative study of the effects of different sulfa drugs (Ancel and Lallemand 1942) demonstrated a wide range of response (Table 3). Micromelia was the most constant feature, parrot beak occurred

TABLE 3

VARIATIONS IN RESPONSE TO DIFFERENT SULFA DRUGS AFTER TREATMENT AT 48 HOURS AND WITH THAT DOSE WHICH GAVE THE HIGHEST RESPONSE. SUSSEX EMBRYOS. DATA OF ANCEL AND LALLEMAND (1942). SIZE OF SAMPLES NOT GIVEN. THE FIGURES FOR INCIDENCE OF ABNORMALITIES PRESUMABLY ARE BASED ON THE FREQUENCY OF MICROMELIA.

Compound	Dose mg.	Abnormalities induced	Total incidence of abnormalities %
Sulfanilamide	1.25	Micromelia, parrot beak, syndactylism	100
N-sulfanilylacetamide (Albucid)	7.5	Micromelia, parrot beak, syndactylism	100
Sodium 1-phenyl-3-p-sulfamylanilino-1, 3-propanedisulfonate (Soluseptacine)	9	Micromelia, parrot beak, syndactylism	57
Sulfapyridine	4	Micromelia, parrot beak	10
Sodium salt of formaldehyde sulfoxylate of sulfanilamide (Pulmorex)	4.15	Micromelia	10
Neoprontosil	12.5	Micromelia	15

only in the presence of micromelia, and syndactylism was only found in embryos which showed the two other traits. The less teratogenic effect a certain compound had, the more were the embryonic responses limited to a stunting of the extremities. This pattern of response does not hold for other substances, such as insulin, which induce micromelia and beak defects.

The period during which insulin treatment leads to rumplessness of chicken embryos is followed by another one in which the embryos respond with micromelia and abnormalities of the beak (Landauer 1947b). The time of maximum effectiveness is near the end of the fifth and the beginning of the sixth day of development. With White Leghorn stock and insulin treatment at 120 hours about 47 percent

of the survivors of the seventeenth day showed micromelia after 2 units of insulin and about 87 percent after 5 units. The maximum incidence of beak abnormalities (i.e. disturbances in formation of the maxillae and other bones of the skull) in White Leghorn embryos was 22.6 percent with 2 units (120 hours) and 55 percent with 5 units (96 hours) of the hormone. The most common beak defect was a shortening of the upper beak; especially after treatment during the fourth day, however, the parrot-beak type of abnormality occurred nearly as often. During the period of maximum susceptibility abnormalities of the extremities and the beak usually coexist, but either one may occur in the absence of the other, although micromelia without beak abnormality was found more often than the reverse situation. The developmental stages during which micromelia and beak defects can be induced with insulin do not coincide completely, the period for beak abnormalities beginning somewhat earlier and tending to end sooner than that for micromelia.

The degree of response of four and five-day chicken embryos to insulin depends on the genotype of the stock from which the eggs came. Breed variations in response seem, however, to be less extreme at this time than during the earlier period when insulin induces rumplessness. Two sets of observations are of particular interest in regard to genetic response differences. It has already been mentioned that embryos of Creeper fowl (whether genetically normal or Creeper) and of the family which is homozygous for, but had been selected against the expression of, the "short upper beak" lethal show high resistance toward the rumplessness-inducing effect of insulin. These stocks also are rather refractory toward insulin in the stages during which White Leghorn embryos give a high incidence of micromelia and beak abnormalities, except that in the "short upper beak" stock insulin leads in a large proportion of treated embryos to shortening of the upper beak. Our observations suggest, therefore, that modifying genes which tend to minimize the morphological expression of hereditary micromelia also afford considerable protection against insulin-induced skeletal defects (micromelia and beak abnormalities as well as rumplessness).

The degree of teratogenic effect of insulin at one period of development cannot be used, however, as a reliable indicator of the extent of insulin-susceptibility of the same embryos at another stage of

incubation. Our high and low response stocks of White Leghorns differ very markedly in the frequencies of rumplessness after insulin treatment during early developmental stages, but show little, if any, dissimilarity with regard to the production of micromelia and beak abnormalities when insulin is injected during the fourth or fifth day of incubation. Similarly, the high insulin resistance of Jungle fowl embryos in early stages disappears later. The results of the injection of 5 units of insulin at 120 hours of incubation into Leghorn and Jungle fowl eggs and into eggs of reciprocal crosses between these two breeds are shown in table 4. The higher incidence of all types of

TABLE 4

THE RESULTS OF 5 UNITS OF INSULIN INJECTED AT THE AGE OF 120 HOURS INTO EGGS OF LEGHORN AND JUNGLE FOWL AND OF RECIPROCAL CROSSES BETWEEN THE TWO BREEDS. DATA IN PERCENT OF SURVIVORS OF SEVENTEENTH DAY OF INCUBATION.

Mating	Micromelia	Beak abnormalities	Eye abnormalities
White Leghorn	87.3 ± 1.8	39.2 ± 2.7	4.3 ± 1.1
Leghorn ♀ x Jungle ♂	78.7 ± 2.7	31.3 ± 2.1	3.0 ± 1.1
Jungle ♀ x Leghorn ♂	96.2 ± 1.3	35.7 ± 3.1	8.9 ± 1.9
Jungle	96.5 ± 2.0	62.4 ± 5.3	9.4 ± 3.2

abnormalities among embryos of Jungle fowl mothers may be ascribed to the smaller egg size and, therefore, relatively larger dose of insulin injected into these eggs. There is little, if any, indication of a maternal effect on response to insulin. The relatively low incidence of beak abnormalities in both crosses may be a result of heterosis.

Insulin treatment in early developmental stages sometimes leads to the occurrence of *polydactylism* in non-polydactylous (White Leghorn) stock. Such chicks may morphologically resemble the standard polydactylous, the duplicate or the diplopod phenotypes of hereditary polydactylism. Since the incidence of such insulin-induced polydactylism is low and the conditions of its appearance are not yet understood we shall not discuss it here. The effect of insulin on hereditary polydactylism, on the other hand, is of considerable interest.

It was mentioned earlier that hereditary polydactylism of the Houdan or Dorking type has many variants deviating from the presence of equally well developed supernumerary fifth toes on each foot. Purebred stock generally breeds reasonably true, but after out-

crosses variability is usually greatly increased. Some of the aberrant phenotypes occur commonly only in heterozygotes, others are found in hetero- and homozygotes. The incidence of all these conditions can be increased or suppressed by selection, though the ease with which this can be accomplished varies greatly. If genetically polydactylous embryos in the course of the fourth day of development are treated with insulin, there is a great increase in the frequency of those aberrant phenotypes which the particular stock tends to produce most commonly (Landauer 1948a). Insulin induces in this material, therefore, phenotypic results very similar to those of genetic modifiers. The extent of these effects is illustrated by the figures in table 5. The

TABLE 5.

DATA COMPARING THE DEVIATIONS FROM BILATERAL POLYDACTYLISM AMONG CONTROLS AND AFTER INSULIN TREATMENT IN THREE DIFFERENT TYPES OF MATINGS. THE INSULIN DATA ARE COMBINED RESULTS OF INJECTIONS AFTER 72 AND 96 HOURS OF INCUBATION AND WITH 2 OR 5 UNITS OF INSULIN.

Mating	Deviations from bilateral polydactylism		Excess produced by insulin treatment
	Untreated control	Insulin-treated	
	%	%	%
F_1 (Leghorn × outbred homozygous polydactylous)	12.0±2.1	55.8±2.5	43.8±3.3
Outbred homozygous polydactylous inter se	10.7±3.1	64.6±5.3	53.9±6.1
Dorking inter se	5.9±1.6	26.2±2.6	20.3±3.1

principle point of these data is that the extent to which insulin interferes with the development of the polydactylous condition is largely determined by the presence or absence of genes which breeders seem to have eliminated from polydactylous stocks because of their interference with the expression of polydactylism. The interaction and potentiating effect of modifiers and insulin are illustrated by a comparison of the results on purebred Dorking stock with those from the two matings of outbred origin.

DISCUSSION

Let us now turn to a more general discussion of our observations and of their bearing on problems of development and heredity.

All the mutations which were described have primarily skeletal manifestations. They also have the common feature that those parts of the skeleton which are affected are of cartilaginous origin, whereas

bones with intramembranous ossification generally remain normal. We know that in some of these conditions, e.g. the long bones of certain types of hereditary micromelia, structure and multiplication of cartilage cells are grossly abnormal. We know also, however, that in all the mutants which have been studied adequately the origin of the defects is to be found in pre-cartilaginous stages. Thus, it has been shown (Rudnick 1945) that the abnormalities of Creeper limbs are already determined before discrete limb regions can be seen in the embryo. In complete or intermediate rumplessness, whether of the dominant or recessive type, the processes leading to tail abnormalities similarly have their inception much prior to the formation of cartilage. A somewhat similar statement could be made about the time of genetic determination of polydactylism. It is clear, therefore, that in all these cases the decisive deviations from normal development occur long before the appearance of those tissues for which the genes in question appear to be specific. It may well be, of course, that the activity of the mutant genes continues to be a decisive factor in the further development and differentiation of the affected parts.

All the morphological abnormalities which we have been able to produce by insulin treatment, with the exception of certain eye defects (bupthalmia) which are probably of a secondary nature, involve parts of the skeleton (skull, long bones, toes, caudal vertebrae, other parts of the vertebral column and ribs). There is much evidence from various sources for the inference that insulin produces its metabolic effects within a very brief period after its introduction into an organism. It cannot yet be decided, however, whether these metabolic changes impinge immediately and directly upon precursors of those parts which later become abnormal or whether the teratological end-results are mediated indirectly. In the case of insulin-induced rumplessness it is likely that the tail abnormalities frequently are already under way before an effective circulation is established. One may suspect, therefore, that insulin leads to local alterations in the tail primordium. Insulin-induced micromelia, on the other hand, is associated with hypoglycemia (Zwilling 1948) whose possible causative role remains to be decided. It has been noted earlier that the appendicular abnormalities of Creeper embryos are determined prior to the existence of a vascular system, a fact which rules out the attempt by Cairns (1941) to explain the Creeper abnormalities on the basis of

defective yolk sac circulation. The same need not be true, of course, for insulin-induced micromelia. In any event, it is interesting to note that the period during which micromelia can be produced by insulin treatment is remarkably long. It begins at about the time when the very first signs of limb buds make their appearance (64 hours) and extends well beyond the completion of cartilaginous primordia of the long bones (168 hours and later). If the metabolic effects of insulin are of a similar nature during this whole period, as seems likely, then it follows that a specific insulin sensitivity persists from the earliest mesenchyme condensations for limb bud formation until at least as late as the early stages of the cartilaginous limb skeleton. This would presumably imply that similar physiological events are of particular significance for limb formation during all these stages. As was suggested earlier, a comparable situation may well exist in the case of hereditary micromelias.

It can obviously be concluded from our observations that insulin has a high degree of specificity for the production of skeletal abnormalities. The site of defect varies with the developmental stage at the time of treatment, e.g. rumplessness early and micromelia late, but—as has just been shown for micromelia—similar results may follow treatment of precursors of certain parts or of the early primordia of these parts. Furthermore, the effect may extend from complete suppression of a part (rumplessness) to more or less severe interference with its formation and growth (micromelia), and to changes in its morphogenetic pattern (polydactylism). The effects of insulin can only be called "tissue specific" in the meaning of Grüneberg (1947) if the term "tissue" is applied to all stages in the ontogenetic history of the cartilaginous skeleton from its earliest beginnings. This tissue specificity is presumably based on certain chemical events for which the tissues in question are peculiar in a quantitative or qualitative manner.

There is no question that different tissues or their precursors react selectively to such substances as insulin and that their reactivity varies with developmental time. In this respect the effect of insulin is similar to that of the mutant genes which we have discussed. The results of using different amounts of insulin at a given embryonic stage are somewhat more difficult to interpret. The most conspicuous consequences of dosage differences are variations in the incidence of particular

abnormalities, i.e, with a higher dose the resistance of a greater percentage of embryos is overcome, in genetic terms the penetrance is increased. In some instances there is a definite relationship between dosage and expressivity, i.e. the degree of abnormality of certain parts. This is especially true for micromelia which after high doses of insulin frequently approaches a phocomelic appearance. At the same time the abnormalities tend to spread to other parts of the skeleton, e.g. in rumpless embryos to the ribs or to vertebrae in more anterior parts of the vertebral column. In this respect the dosage effects of insulin are comparable to some of the differences found between heterozygotes and homozygotes of certain mutants, such as the micromelias of Creeper and Cornish fowl.

Modifications of mutant types which are accomplished by modifying genes, such as the intermediate forms of the two rumpless mutations, cannot be duplicated in the insulin experiments by using small doses of the hormone. Thus, tests involving insulin dosage differences up to one hundredfold (between 0.05 and 5 units, injected prior to incubation) did not reveal a significant trend in the proportions of intermediate rumpless embryos which were produced (White Leghorn stock). It appears that for such modification of the insulin effect the presence of certain modifying genes is as necessary as in the case of the mutants.

The manner in which a mutant gene impresses its effects upon different parts of developing embryos is influenced by factors of the normal pattern of development. This may be illustrated by the following examples. In all cases of hereditary micromelia the leg bones are much more affected than the wing bones. During embryonic development (in contradistinction to the post-hatching period) the normal rate of growth of the leg bones is much higher than that of the wing bones (Streich and Swetosarow 1937). Within both pairs of extremities, but more clearly in the legs, two gradients are always found which reflect the extent to which the mutant genes interfere with normal growth of the long bones. There is a proximo-distal gradient of increasing reduction in relative length. This may indicate that the sequence of formation of the bones is an important factor or it may be a consequence of the fact that in normal development an increasing gradient of negative heterogony exists along the axis of the extremities (Landauer 1934; Lerner 1936, 1937). This gradient is reflected in

the chemical composition of the long bones (Tadokoro 1930). There is also a parallelism, however, between the prospective absolute length of a bone in normal development and the extent of its relative reduction in micromelia, the higher the inherent normal growth capacity of a bone the greater being its proportionate reduction (Landauer 1934). The inverse relation between normal growth characteristics and the degree of their modification in micromelia can also be clearly demonstrated by a comparison of our data with those for normal growth of other birds, e.g. the observations of Huggins (1940) on wrens. These features of hereditary disproportionate dwarfism are equally characteristic for insulin-induced micromelia. Parallel gradients of relative disturbance are also found in the two rumpless mutants and in insulin-induced rumplessness. It seems clear, therefore, that the degree of expression of mutant genes as well as of abnormalities imposed by external agencies depends upon the inherent growth characteristics of the affected parts. The modulating influence of these growth patterns is similar in hereditary and insulin-induced abnormalities. It should be added in passing that a general retardation of embryonic growth need not lead to specific abnormalities. It has been shown, for instance, that adrenal cortex extract is a potent inhibitor of general body growth of chicken embryos without giving rise to disproportions or other defects. However, in combination with insulin adrenal cortex extract exaggerates the incidence as well as the degree of expression of micromelia (Landauer 1947c). It is evident, therefore, that a general growth retardation may predispose toward exaggerated response to teratogenic agents. Mutants may have similar effects, e.g. the heightened teratogenic action of selenium in Creeper embryos (Landauer 1940).

Another feature which may throw some light on the developmental factors governing the expression of hereditary and experimentally-produced skeletal abnormalities is the occurrence of certain asymmetries. In the early lethal type of homozygous Creeper embryos the left eye generally is much more poorly developed than the right one (Rudnick and Hamburger 1940, Cairns 1941). Gruenwald (1944) has shown that the Creeper condition only exaggerates an asymmetry which occurs in the majority of normal embryos. In a heterotactic embryo he observed the reverse situation, i.e. smaller size of the right eye. It is also known that sporadic microphthalmia has a much higher

incidence on the left than on the right side (Landauer 1938, Gruenwald 1944). The temporary normal asymmetry of eye size disappears, according to Gruenwald, shortly after the establishment of an effective circulation, and Gruenwald assumed that the passing size inequalities are due to differences in oxygen supply after the head has turned on to its left side, and that debilitating factors may render the asymmetries permanent. An alternative explanation will be set forth in the following discussion.

There are many other examples for the preferential involvement of one body side in certain abnormalities. For instance, among malformations which are produced by the presence of selenium in eggs the wings as well as the eyes are more frequently abnormal on the left side, whereas the opposite, i.e. higher incidence of abnormalities on the right side, is found in the legs (Landauer 1940). Or, in the scoliosis of recessive rumplessness the convexity tends to be toward the left side (Landauer 1945a). But of greatest interest for our present argument is the fact that unilateral polydactylism, a common heterozygous expression of the gene for polydactylism, occurs preponderantly on the left foot and only rarely on the right one, and that it is possible to increase the incidence of either one of these asymmetry types by selection.

It seems reasonable to assume that there is a common cause of all these tendencies for asymmetrical expression of effects imposed either by genes or by forces of the external environment. Indications for the existence of lateral reaction differences between the two body sides exist already in the head process stage (i.e. prior to turning of the embryo on to its side) as shown by dissimilar potentialities of grafted fragments (Rawles 1936). They are still present in adult fowl as is demonstrated by lateral differences in fresh weight of the long bones (Weakley and Dustman 1939) and by asymmetry of moulting pattern (Juhn 1947). These observations, together with the fact that the incidence of one or the other type of heterodactylism can be changed by selection, indicate that we are dealing with an intra-embryonic agency.

It has been shown earlier that in polydactylous embryos the incidence of heterodactylism can be raised by insulin treatment. As in unselected heterozygous polydactylous stock, these cases of insulin-induced unilateral polydactylism occur predominantly on the left foot. There are also agencies, however, which seem to favor the appearance

of heterodactylism of the right foot. This is shown by the following figures which give the incidence of aberrant forms of polydactylism among embryos with sporadic rumplessness in a mating of Dorking fowl (homozygous for polydactylism). The extremely high incidence of sporadic rumplessness in this mating (11.0%) was probably due

Tail	Number of embryos and chicks	Incidence of different phenotypes in percent				
		Bilateral polydac-tylism	Left 5 right 4	Left 4 right 5	Left 4 right 4	Ectrodac-tylism
Normal	785	98.9	1.0	0	0.1	0
Rumpless	97	89.7	0	7.2	2.1	1.0

to the presence of a virus infection (Newcastle disease). But what is of interest here is the fact that the rumpless embryos, but not the normal ones, had among them a relatively high percentage of the rare form of heterodactylism with five toes on the right foot. The same set of circumstances presumably led in this material to the occurrence of rumplessness and, with lower frequency, to heterodactylism or complete suppression of the polydactylous condition. It seems probable, therefore, that we are dealing with a mechanism which has a general effect on embryonic development with repercussions in particular parts. In an earlier discussion (Landauer 1948a) it was suggested that the mechanism in question resides in the pattern of local growth rate differentials, and that this pattern modifies the expression of many developmental abnormalities, whether of genetic or extrinsic origin. This is still, I believe, the most probable explanation.

In addition to lateral differences in manifestation our observations on hereditary polydactylism have produced evidence for the existence of modifying factors which affect the two pairs of extremities independently (Baumann and Landauer 1944, Landauer 1948a). In discussing similar material Schmalhausen (1934) expressed the opinion that it is "inadmissible" to assume the existence of separate modifiers for wings and legs. This was probably a reflection of the dilemma which many embryologists and geneticists have seen in the spurious alternative between regional differentiation and universal presence of the genome in all body cells.

Growth rates may also play a role in the "maternal" inheritance of the rumplessness-response to insulin as it has been demonstrated for

reciprocal crosses between Jungle and Leghorn fowl. The investigations of Tennent (1914, 1922) and A. R. Moore on echinoderms, of J. A. Moore (1941, 1946) on frog hybrids and the recent work of Khesin (1948) on Drosophila have demonstrated in widely different classes of animals that the early rates of embryonic development are chiefly, if not exclusively, determined by maternal factors. Rate factors of this type may govern the rumpless-response to insulin in our Jungle × Leghorn crosses, but do not exist any longer during the later period when insulin induces micromelia.

The causes of the maternal inheritance in our insulin experiments (as, indeed, the features of developmental rate) may trace back either to properties of the egg cytoplasm or to effects of the maternal chromosomes within the egg. In reciprocal crosses between Creeper and normal fowl it was possible to ascribe a maternal influence on embryonic mortality to abnormalities of egg shell formation by Creeper hens (Landauer and Bliss 1943). This is a maternal effect sensu stricto. In most instances, however, it has not been possible to distinguish between maternal influences on structure and composition of egg cytoplasm or membranes and "pre-zygotic" effects of the maternal chromosomes of the egg on early development. Both agencies may be of great importance in setting the stage upon which the zygotic nuclei later play their roles. Which of them is decisive for the observed differences in response to insulin remains unknown.

In addition to the factors which are responsible for asymmetries and the maternal influences we must consider briefly the role of modifying genes in the phenotypic manifestation of skeletal abnormalities. This is not to say, of course, that the former agencies are not also under genetic control. The role which modifiers play in poultry genetics can hardly be overestimated. I know of no hereditary trait of fowl, whether dominant or recessive, which is not under the influence of multiple modifying genes. This is by no means a situation peculiar to poultry, as is witnessed by such (not very satisfactory) terms as "dominigenes" and "polygenes." From a study of any genetic trait of poultry which has been analyzed in some detail one is led to Harland's (1939) conclusion that "the genetical architecture of species —may be viewed as great systems of co-ordinated modifiers." This concept implies that all the important physiological functions of organisms in the course of their evolutionary history have been built up by

a network of interlocking and often mutually reinforcing genes and that the conspicuous mutants usually are genetic accidents which have produced breaks in this network with consequent quantitative or qualitative changes in specific functions.

Because the absence of symptoms is the definition of normality, the complexity of the normal condition is frequently overlooked. It is more than a simile to illustrate this situation with observations on skeletal material. The complex internal architecture of a long bone may be modified by genes (chondrodystrophy) or by external agencies (mineral or vitamin deficiencies) which either reduce the number of trabeculae or are responsible for the formation of additional ones (estrogen) without affecting stability. Rearrangements occur only in response to important changes in stress. Under such abnormal conditions the resources of repair and adjustment become apparent. These resources may vary greatly from individual to individual. Their significance lies in the latitude of possible equilibration under adverse conditions. In the developing organism the genome represents this complex pattern of equilibrating forces some of which are likely to come into action as modifying genes whenever there is danger of a disequilibrium in its integrated activities.

The presence or absence of an effect by modifiers of certain mutants on their chemically-induced "phenocopies" should furnish some information concerning the physiological relatedness of the genes and the chemicals in question. A positive effect of modifying genes on the expression of chemically-induced abnormalities would seem to furnish direct proof for similarities of chemical action. A negative result is more difficult to interpret since it is always possible that the conditions imposed by a chemical include additional effects which prevent the modifiers from their normal activity.

We have been unable to secure any evidence for an effect by the modifiers of the two rumpless genes upon the expression of insulin-induced rumplessness, but adequate tests have not been made with the recessive rumpless stock. On the other hand, our data indicate clearly that the accumulation of modifiers against expression of micromelia and beak abnormalities in a stock homozygous for the "short upper beak" lethal furnished a high degree of resistance against induction of micromelia by insulin. Experiments with an outbred stock of Creeper fowl, known to carry modifiers of the micromelic condition,

also provided evidence for a high degree of protection against the usual teratological action of insulin. Both of these stocks also enjoy a considerable measure of protection against the induction of rumplessness by insulin.

Our observations on Jungle fowl may be of some interest in this context. Our stock of Jungle fowl is singularly free from sporadically-occurring developmental abnormalities, such as rumplessness, eye defects, beak abnormalities, and so forth. This and the very low sensitivity of Jungle fowl embryos to insulin during early stages of development point to the existence of highly effective equilibrating mechanisms in this material. The fact that various mutants show, after their transfer to Jungle stock, a reduced degree of dominance (Fisher 1935, 1938) may have a similar basis. The many drastic changes, such as great differences in body size, which occurred in the production of the domestic breeds of fowl may well have led to a lowering in the effectiveness of the originally available multiple developmental safeguards.

We come finally to the important question of what the experiments with insulin and similar investigations can teach us about the nature of certain mutants and about the fundamental processes with which either mutant genes or external agencies interfere. Extensive experiments, especially with Drosophila, have demonstrated that a great number of non-hereditary modifications can be produced by exposing developing organisms to such physical agents as high or low temperature, x-rays and other radiations. Such modifications are now generally referred to as phenocopies. Little attention has, on the other hand, been paid to the production of such modifications by chemical means. As far as animals are concerned work of this nature seems to be limited to the experiments of Rapoport (1939, 1940, 1947), Di Stefano (1943), Gersh (1946), Gloor (1946, 1947), Vogt (1947), Macdonald (1948) and Niggli (1948) on Drosophila and the experiments with chicken embryos which have just been reviewed.

It is obvious, of course, that exposure of embryos to x-rays or extremes of temperature has drastic inhibitory effects. The beautiful x-ray experiments of Wolff (1936) on chicken embryos have shown, for instance, that certain parts or organs can be eliminated selectively, and the resulting abnormalities may, as in the case of rumplessness, closely resemble mutant forms. It is unquestionably impressive that so many different mutant types can be "copied" by the application

of physical agencies. The one conclusion which, I think, one can draw from this is that the majority of mutants produce their phenotypic effects by disrupting or inhibiting normal gene activities.

The mere fact, however, that non-genetic modifications grossly resemble mutant forms, even if the similarity is great, cannot be a sound basis for extrapolating to similarity in causation. A comparison of the time of gene action (which, however, may and in some instances certainly does appreciably precede visible symptoms) with the developmental period during which modifications can be produced experimentally may indicate whether or not there is any reason for suspecting physiological relatedness. Thus, Henke, Fink and Ma (1941) concluded that certain heat modifications in Drosophila, though similar to mutants, could not be their phenocopies because their temperature-sensitive periods were later than the "phenocritical" periods of the mutant genes. They rightly pointed out further that in those cases in which these periods coincide the modifications *may* but need not be phenocopies (see also discussion in Henke 1947).

If the term "phenocopy" is to indicate similarity in the fundamental reaction chains which lead to the appearance of a genetic trait or a non-hereditary modification, and this we believe to be the only useful sense of the term, then we need in each instance specific proofs for such similarity. It is well known that adult phenotypes which may be virtually indistinguishable can be brought about by separate mutant genes through developmental events which have little in common. Dominant and recessive rumplessness or different genetic forms of taillessness in mice are cases in point. Another example is the fact that *within breeds* the tendencies to produce sporadic rumplessness or to respond to insulin with rumplessness show no indication of association (χ^2 in three analyzed groups 0.116, 2.665 and 0.011, respectively), although in both cases the incidence is subject to change by selection and differs between breeds. Mutants with very similar heterozygous phenotypes but quite distinct homozygous forms of expression illustrate also the danger of assuming similar developmental histories on account of phenotypic resemblance. Goldschmidt (1935) argued that, in the case of his temperature modifications (phenocopies) in Drosophila, it could not be assumed that this one agency (viz., heat) had specifically led to many different qualitative changes in chemical reactions. In view of the complexity of metabolic events during embryonic develop-

ment I see no difficulty in this respect. Moreover, it is clear that identical qualitative changes may at different stages lead to dissimilar results.

There is no reason to doubt that "changing the relative rates of integrated developmental processes" (Goldschmidt 1945) may often be the way in which mutant genes and extrinsic modifying agencies produce their results. But, in view of the dearth of relevant information, there is little justification to exclude interference with definite steps of chemical synthesis as an equally likely source of variation. In fact, I see at present no way to decide whether the great majority of cases are of a quantitative or a qualitative nature or the result of a combination of both.

We believe that at least a part of those cases of insulin-induced rumplessness which survive to late stages are true phenocopies of hereditary rumplessness. There is strong indirect evidence in the same direction for the insulin-induced and genetic modifications of polydactylism. In the case of micromelia the effectiveness of the same modifiers on mutants and insulin modifications points to a similar conclusion but direct developmental and histological comparisons have not yet been made.

Such evidence as we have been able to secure encourages the conviction that the study of chemically produced phenocopies may become an important branch of physiological genetics. These methods will, of course, have certain limitations. In most cases, for instance, chemical interference will only become possible after a particular reaction chain has been initiated, whereas a mutant gene may prevent the initial steps from occurring. In most cases it will presumably be difficult to duplicate gene effects quantitatively or to use chemicals in such a way that secondary effects are avoided. Obstacles of this nature may often account for difficulties in securing exact phenocopies.

With such limitations in mind it should be possible to approach a great variety of problems. Among these the biochemical nature of the experimental conditions is the most important one. In our material Zwilling (1948) has found that insulin produces hypoglycemia in very early embryos and that there is a parallelism between degree and duration of hypoglycemia on the one hand and extent of micromelia on the other. Does this parallelism point to causal relationship and to disturbances of carbohydrate metabolism as the origin of our pheno-

copies? We believe that we have evidence in favor of this conclusion (Landauer, 1948c).

It should be possible with chemical phenocopy methods to trace the initiation of gene effects to earlier stages than is possible visually and to localize these effects more accurately. It should also be possible to study means of counteracting the chemical induction of phenocopies. Positive results in this respect would open the way for a direct comparison of the biochemical effects of mutant genes and phenocopies. Thus we may hope eventually to obtain information about the manner in which mutant genes contribute to or interfere with synthetic processes which decide the fate of particular tissues and organs.

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